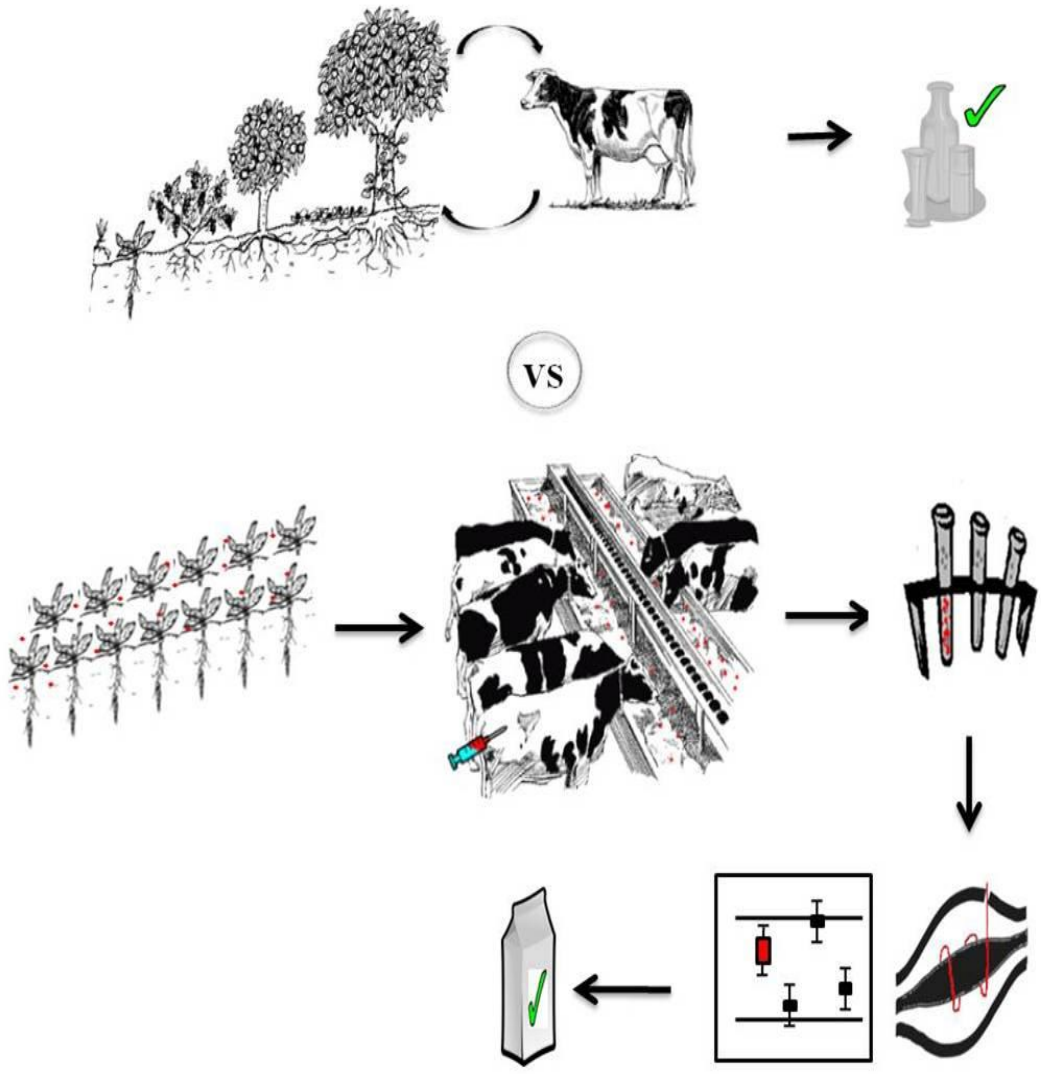
Methodologies for the analysis of veterinary drugs and growth promoters in the scope of food safety control



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Methodologies for the analysis of veterinary drugs and growth promoters in the scope of food safety control

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Methodologies for the analysis of veterinary drugs and growth promoters in the scope of food safety control

Doctoral Program:
Química Analítica del Medi Ambient i la Pollució

PhD thesis
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ATTESTS

The current PhD thesis work titled,

Methodologies for the analysis of veterinary drugs and growth promoters in the scope of food safety control

has been conducted by Praveen Kumar, K in the Department of Analytical Chemistry at University of Barcelona, under my supervision.

Barcelona, 7 March 2014

Dedicated to my father,
Dr.M.N.Kumar

Acknowledgement

I started this doctorate in philosophy (Greek: philosophia – “love for wisdom”) journey for my love for food and hunger for wisdom.

This journey gave me a chance to meditatively see food safety through various lenses: technological advances, legal framework, personnel at various levels, institutes with various specializations and societal implications.

On the first hand, I express my gratitude to the wonderful team – R.Companyó, A.Rubies and F.Centrich and the collaborative research environment created by them. This collaboration between and UB and ASPB helped to set necessity based research purposes (goals) and develop productive processes to achieve them. I spent most of my thesis time at ASPB and I owe a special thanks to A.Rubies for his mentorship, help with instrumentation and inputs about food safety control. I thank Nuria Cortes and Josep Caixach for their inputs to build my foundation in high resolution and accurate mass spectrometry. I thank J.Hollender and Uchem research group for the exciting trip in non targeted analysis. I thank M.Granados, Marc, Silvia, Carles, Quim at UB, Sonia and Victor at ASPB and many more people to whom I say “Hola” everyday, which ran my days smoothly.

Needless to say, the presence of my wife, Anusha in Barcelona was such a support that without her I might have needed few more years to complete my PhD. A big hug to my mom and my brother for believing in me and taking care of issues back at home.

This stint outside Tamil Nadu, India not only gave me a good foundation for my career, but also increased my cultural quotient (CQ) a lot.

I thank my life and the people who pass by in it for the experiences gained and I hope that would help me to enrich experiences in others lives, and the society I am in.

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Acronyms

ACN	Acetonitrile
ADI	Acceptable Daily Intake
AIF	All Ion Fragmentation
AFSSA	French Agency for Food, Environmental and Occupational Health and Safety
AGC	Automatic Gain Control
APCI	Atmospheric Pressure Chemical Ionization
APPI	Atmospheric Pressure Photo Ionization
ASPB	Public Health Agency of Barcelona
CAC	Codex Alimentarius Commission
CAS	Chemical Abstracts Service
CCα	Decision limit
CCβ	Detection capability
CFSAN	Center for Food Safety and Applied Nutrition
CNA	National Food Centre, Spain
CONTAM	EFSA panel on Contaminants in food chain
CRL	Community Reference Laboratory
CRM	Charge Residue Model
C trap	C shaped ion trap
CVMP	Committee for Medicinal Products for Veterinary Use
DAD	Diode Array Detector
ddMS/MS	Data dependent MS/MS
DDA	Data dependent acquisition
EAWAG	Swiss Federal Institute of Aquatic Science and Technology
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay

EMA	European Medicines Agency
ENAC	Spanish National Accreditation Body
ESI	Electrospray Ionisation
EU	European Union
FAO	Food and Agriculture Organization
FAPAS	Food Analysis Performance Scheme
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
FSSAI	Food Safety and Standards Authority of India
FWHM	Full Width at Half Maximum
GC	Gas Chromatography
HESI	Heated Electrospray Ionisation
HFBA	Heptafluorobutyric acid
HILIC	Hydrophilic Interaction Chromatography
HLB	Hydrophilic Lipophilic Balance
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
IEM	Ion Evaporation Model
IP	Identification Points
IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
IT	Ion injection time
IUPAC	International Union of Pure and Applied Chemistry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JEMRA	Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment
JMPR	Joint FAO/WHO Meeting on Pesticide Residues

LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
LTQ	Linear ion trap
m/z	mass to charge ratio
MAE	Microwave Assisted Extraction
MIP	Molecular Imprinted Polymers
MRL	Maximum Residue Limit
MRM	Multiple Reaction Monitoring
MRPL	Minimum Required Performance Limit
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry
NCE	Normalized Collision Energy
NOAEL	No Observed Adverse Effect Level
NPC	Normal Phase Chromatography
NRL	National Reference Laboratory
OCL	Official Control Laboratory
PCX	Polymeric Cation Exchange
PLE	Pressurized Liquid Extraction
QIT	Quadrupole Ion Trap
Q-Orbitrap	Quadrupole Orbitrap
QqQ	Triple quadrupole mass analyzer
QTOF	Quadrupole Time of Flight
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RF	Radio Frequency
RIVM	National institute for public health and environment, The Netherlands

RPA	Reference Points for Action
RPC	Reverse Phase Chromatography
RT	Retention Time
SFE	Supercritical Fluid Extraction
SPE	Solid Phase Extraction
SIM	Selection Ion Monitoring
SRM	Selected Reaction Monitoring
tMS/MS	Targeted MS/MS
TIC	Total Ion Chromatogram
TOF	Time of Flight
US	United States
UHPLC	Ultra High Performance Liquid Chromatography
UV/VIS	Ultraviolet - Visible spectroscopy
WHO	World Health Organization
XCW	Weak Cation Exchange
XIC	Extracted Ion Chromatogram
ZIC	Zwitterionic

Summary and thesis structure

Veterinary drugs and growth promoters are a part of many chemical hazards that can be found in the modern food chain, and they pose risks to human health such as antimicrobial resistance. The presence of these drug residues increased with intensive farming practices and is an issue of global concern. Food safety authorities around the world establish control programs by scientifically assessing the risk of each drug and set maximum levels for each drug in various matrices. The testing laboratories and analytical methods to test samples from food and feed chain form the core of such programs and provide evidence for regulatory authorities to take decisions. In the drug residue testing, there is a need for improved methods of testing that can provide high selectivity, high throughput, robustness and affordability. In that sense, one main goal of this thesis was to develop analytical methodologies for various families of veterinary drugs and growth promoters in feed, food and biological samples from food producing animals. The choice of analytes and matrices to be tested depended on whether research and innovation is required to develop methods with improved performance characteristics.

First three chapters form the introduction of this thesis. Chapter 1 provides an overview on food safety hazards, food safety authorities, legislations for control of veterinary drugs and growth promoters and the role of analytical laboratories in the control program. Chapter 2 covers sample preparation techniques and the typically used chromatographic (LC) and detection (MS) techniques for drug residue testing. Chapter 3 reviews various applications in literature and focus on trends in developing analytical methodologies for food safety testing which move in the direction of comprehensive analysis of samples owing to very high number of contaminants and increasing number of unknown contaminants.

Chapter 4 presents a method developed and validated for analyzing eight sulphonamides in six types of feed based on liquid chromatography (LC) and ultraviolet detection, carried out at University of Barcelona.

Chapter 5 presents the results from a systematic study of various hydrophilic interaction chromatography (HILIC) stationary phases for analysis of aminoglycosides and also reports two analytical methods based on HILIC- tandem mass spectrometry (MS/MS) for analysis of ten aminoglycosides in honey and animal kidney samples. This work was carried out in collaboration with laboratory of Agencia Salut Publica de Barcelona

(ASPB) and the methods were validated according to Decision 2002/657/EC and implemented for routine analysis of samples in accordance with the requirements of ISO 17025: 2005. The laboratory of ASPB acquired an LC- high resolution mass spectrometry (HRMS) instrument (Q-Orbitrap) and in order to incorporate the instrument for routine targeted analysis, an exploration of various parameters of the equipment was necessary. In that sense, a systematic study to explore various modes of acquisition has been conducted and a method to analyze nine hormones in urine has been transferred to HRMS and is reported in Chapter 6. Gaining understanding about the functioning of the instrument, an improved method to analyze aminoglycosides based on HRMS was developed and reported in Chapter 5.4. Moreover, the LC- HRMS instrument has been incorporated in the laboratory for resolving ambiguous test results. A case study of resolving a false positive result from the analysis of ronidazole in meat was presented in Chapter 7. This case study highlights the pitfalls with low resolution mass spectrometry and existing confirmation criteria for identification with mass spectrometric detection.

Inspired by the potential of HRMS in food safety testing for comprehensive analysis of samples, a research stay was carried out at the laboratory of Prof. Juliane Hollender for 3 months to develop a non targeted screening workflow using various HRMS data mining and analysis tools. This work is presented in Chapter 8.

At the end of this thesis, conclusions and outlook are presented followed by a list of bibliographic literature.

I. Introduction

The right to adequate food is realized if people have access to food that,

- provides sufficient nutritional value and micronutrients for a person to lead a healthy and active life
- **is free of hazardous substances**
- is acceptable within a given culture,

explains the Food and Agriculture Organization (FAO) of the United Nations in "Voluntary guidelines to support the progressive realization of the right to adequate food in the context of national food security" [1].

1. Food safety

In the later 19th and early 20th century, common food safety issues consisted were mainly adulteration issues such as diluting milk and wine with water, bakers adding chalks to whiten bread, adding calcium sulfate and sawdust to increase the weight of bakery products. Although most such food adulteration was harmless, some were poisonous such as adding lead, copper, and many salts to create colour candies to appeal children. Food adulteration was mainly to cheapen products by adding inferior ingredients [2]. Although adulteration is as old as trading, the food adulteration scaled to high levels with the industrial revolution. In the last 50 years, several food safety crises took place and were widely publicized in the media and scientific journals. Table 1.1. summarizes some of the notable food safety hazards in the 20th century.

Table 1.1. Notable food safety hazards in the 20th century.

Year	Issue
1981	**Toxic oil syndrome in Spain**
1986	**Mad cow disease in Britain**
1996	**Salmonella in peanut butter in Australia**
1999	**Dioxin affair in Belgium**
2002	**Acrylamide found in starchy foods cooked at high temperature**
2003	**Pesticides in soft drinks in India**
2008	**Melamine contamination of baby milk formula in China**

2011	Ractopamine, a Beta agonist, in animal feed in China

Moreover with intensive and industrial farming practices, globalization and centralization of food production and supply chains, the type of food safety hazards has changed in the late 20th century. A test sample for food safety in the 20th century may contain many undesired chemicals apart from the adulterants and this has induced transformation in testing laboratories from food composition and adulteration analysis to residue and contaminant analysis.

1.1. Food safety hazards

A hazard is a biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect. The food safety hazards can be classified into three main categories: chemical, microbiological and physical. Since this thesis work is oriented towards the field of chemical food safety, microbiological and physical hazards are not detailed.

The chemical hazards can be introduced in food chain during production, processing, supply, preparation and consumption. Different types of chemical hazards and some examples in each category [2] [3] are given in table 1.2.

Table 1.2. Types of chemical hazards in food safety

Chemical hazards	subcategories
Agrochemical	Pesticides, herbicides, veterinary drugs, hormones, fungicides, fertilizers.
Environmental and industrial contaminants	Heavy metals, Polychlorinated biphenyls, dioxins, polycyclic aromatic hydrocarbons, radionuclides.
Hazards produced during processing and storage	Heat produced chemical hazards (acrylamide, furan, heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, N-nitrosamines, lipid degradation products) Chemical hazards produced during non

	thermal processing and storage (ethyl carbamate)
Packaging-derived hazards	Monomers (vinyl chloride, styrene, acrylonitrile) Pigments (lead) Plasticizers (phthalates) Other (Bisphenol A, semicarbazide)
Allergens	Major food allergens (milk, peanut, egg, tree nut, soy)
Natural toxins	Mycotoxins, plant toxins, marine toxins
Unconventional chemical hazards	Adulterants (melamine)

Although there are numerous chemical hazards, they are not primary health hazards to be found in food. The US Food and Drug Administration (FDA) ranked the relative importance of health hazards associated with food. The ranking of the hazard in descending order is given below [4]. It can be noted that microbial contamination ranks first and the chemical hazards are ranked after microbial hazard.

1. Microbial contamination
2. Inappropriate eating habits
3. Environmental contamination
4. Natural toxic constituents
5. Pesticide residues
6. Food additives

However, according to a public survey the consumers are concerned about chemical hazards in the food. Eurobarometer, a series of public opinion surveys conducted by the European Commission, has reported a survey on food safety risk perception in the EU, in 2010. According to the report from a survey, 19% of the citizens cite chemicals, pesticides and other substances as major concerns to be worried about. In another survey, around 70% of people are worried about pesticide residues in their fruits, vegetables and cereals, and the presence of antibiotic residues/hormones in meat [5].

1.2. Food safety authorities around the world

The occurrences of massive food safety hazards triggered a lot of attention from media and resulted in the loss of consumer confidence [6]. In order to safeguard the public health and assure safety of food from farm to table, many governments in the world started updating their food safety laws and create centralized food safety authorities [7]. In 2002, World Health Organization (WHO) developed a global strategy on the prevention of food borne diseases. This strategy requires holistic, risk-based and timely food safety policies and strategies, which is now being implemented worldwide [8, 9].

Previously, in 1962, WHO and FAO of the United Nations established an international body, Codex Alimentarius Commission (CAC) to protect the health of consumers and to facilitate food trade by setting international food standards (Codex Alimentarius). Codex Alimentarius is a collection of international food standards that are adopted by the CAC, and are recommended to the different government authorities [10].

In 2002, European Commission created European Food Safety Authority (EFSA) combining different information agencies [11]. In 2011, India created Food Safety and Standards Authority of India (FSSAI) under the Food Safety and Standards Act, 2006, which merged and updated different acts related to food safety [12]. Some other prominent food safety authorities and international organizations related to food safety are summarized in table 1.3.

The main role of such regulatory agencies is to manage public health hazards in food by risk analysis approach (Figure 1.1). A risk is a function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard(s) in food. It can be noted that all such national/centralized authorities were developed only after 1950s, to combat with the rising food safety issues.

The risk analysis consists of risk assessment, risk management and risk communication

Table 1.3. Significant food safety authorities, organizations and laws around the world

Organization/Law	Country	Year
National		
Food safety and standards authority of India (FSSAI)	India	2011
Food safety law	China	2009
Food Safety Commission	Japan	2003
European Food safety Authority (EFSA)	Europe	2002
FDA Food Safety Modernization Act	USA	2011
FDA Center for Food Safety and Nutrition (CFSAN)	USA	1984
USDA Food safety and inspection service (FSIS)	USA	1977
Global		
Codex Alimentarius Commission (CAC)		1962
World Health Organization (WHO)		1948
Food and Agriculture Organization (FAO)		1945

Figure 1.1. Risk analysis framework by Codex Alimentarius adapted from the webpage of WHO [13]

Risk assessment involve,

1. Hazard identification – identification of potential health effects associated with a particular agent (biological, chemical or physical).
2. Hazard characterization – the qualitative and quantitative evaluation with biological, chemical and physical agents which may be present in food, involving dose response assessments
3. Exposure assessment – qualitative and quantitative evaluation of the degree of intake likely to occur, such as establishing toxicological limits based on dietary intake patterns.
4. Risk characterization – integration of hazard identification, hazard characterization and exposure assessment into an estimation of adverse effects in a given population

Joint FAO/WHO Expert Committee on Food Additives (JECFA) for food additives, contaminants and veterinary drugs and the Joint FAO/WHO Meetings on Pesticide Residues (JMPR) assess the risks of chemicals in food. Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (JEMRA) addresses the risk assessment of microbiological hazards.

Risk management is the process of weighing policy, based on risk assessment results and select appropriate control measures such as monitoring contaminants in food through control laboratories.

Risk communication is an interactive process of information exchange on risk among risk assessors, risk managers and other interested parties [14, 15].

Although such extensive mechanisms exist in current established food safety policies, the current risk management practices are sometimes criticized. For example, an acrylamide crisis was first broke at Hallandsas, Sweden in 1997. It took 6 years to set maximum residue limits (MRL) for the presence of acrylamide, a genotoxic carcinogen in drinking water [16]. In the similar way, in case of dioxin crisis, which first broke on 1999, European Union (EU) MRL was set only on 2001 after two years [17]. In another case example, Diethylstilbesterol, a hormonally active substance has been used for growth promotion since 1950s and concern over its carcinogenicity raised in 1970s, which led to a ban on the use of this compound at European level only in 1987, after

many years of usage [18]. SAFE FOODS, an European project established in 2004, studies the challenges in current risk management practices and addresses the issues found. The framework recommends three main changes in the risk analysis approach to improve transparency, openness and accountability. Three changes in the risk analysis approach are : a) including framing stage, where interested parties, officials work together to gain initial understanding of the issue, set objectives for regulatory action, b) increase the scope of risk assessment, including development of genomic, proteomic and metabolomic profiling methods c) add evaluation stage to evaluate the risks, costs and benefits [19]. On the other hand, centralized food safety authorities established recently in large countries like India and China face different kind of challenges. Extensive laboratory networks that require huge costs, lack of skilled personnel and high number of samples to be analyzed are some of the important challenges [20, 21]

1.3. Veterinary drugs and legislatory framework

1.3.1. Veterinary drugs and their use

In modern intensive agricultural practices, veterinary drugs are used in large scale as feed additives or through drinking water to prevent outbreak of diseases or for treating purposes. Also, growth promoting agents are administered illegally to stimulate growth of the animals [22]. In the EU, the use of antimicrobials as growth promoters has been banned. The misuse of antimicrobials leads to the risk of antimicrobial resistance, which is a natural phenomenon. Antimicrobial resistance is resistance of microorganism to an antimicrobial medicine to which it was originally sensitive. Resistant organisms (bacteria, fungi, virus and some parasites) can withstand attack by antimicrobial medicines like antibiotics, antifungals, antivirals, and antimalarials, so that infections persist and increasing risk of spread to others. Antimicrobial resistance is a global concern as there are only few new drugs under development to combat against resistant infections. The classification of drugs and their use are summarized in the following table 1.4.

Table 1.4. Summary of veterinary drug classes and theirs uses.

Drug classes	**Use/Comments**
Anthelmintics	Used against intestinal worms, lungworms and liver fluke. Frequently used compound groups are benzimidazoles and

	ivermectins
Antibiotics	Used in general for prophylactic/treatment purpose.
• Aminoglycosides	Used against gram negative bacterial infections. Aminoglycosides show nephrotoxicity and ototoxicity.
• Beta – Lactams	Active against gram positive and negative bacteria. Beta lactams – penicillins and cephalosporins are the most commonly used antibiotics.
• Macrolides	Active against gram positive bacteria and are used to treat respiratory diseases.
• Peptides	Active against bacteria, viruses and eukaryotic pathogens. Metabolize more readily than small molecules.
• Sulfonamides and trimethoprim	Are bacteriostatics used against gram positive and negative bacteria. Trimethoprim with sulphonamides show synergistic effects. Show carcinogenicity.
• Tetracyclines	Used against gram positive and negative bacteria
• Quinolones	Used against gram positive and negative bacteria
• Chloramphenicol	Used against gram positive and negative bacteria

• Malachite green	Used to treat ectoparasites in aquaculture.
Coccidiostats - Ionophores, nitroimidazoles and nitrofurans	Used to prevent and treat coccidiosis
Hormones • Anabolic steroids	Administered to animals to increase the rate of growth.
• Corticosteroids	Used as anti inflammatory drugs.
• Thyreostats	Inhibit thyroid function and reduce the circulation of thyroid hormones. This results in increased filling of gastro intestinal tract and increased water retention by the animal, therefore gaining weight.
Beta agonists	Used to treat respiratory diseases. These drugs accumulate in retina of calves, pigs and turkeys.
Tranquilizers	Administered to animals before transporting to market as stress in animals can deteriorate meat quality.

1.3.2. Maximum Residue Limits and control system for veterinary drugs

In the EU, the council directive 96/23 EC [23], lays down the measures to monitor, veterinary medicines, pesticides and contaminants in food of animal origin. This directive groups unauthorized substances and substances with anabolic effect as group A and the veterinary drugs and contaminants as group B as in figure 1.2.

GROUP A — **Substances having anabolic effect and unauthorized substances**

(1) Stilbenes, stilbene derivatives, and their salts and esters

(2) Antithyroid agents

(3) Steroids

(4) Resorcylic acid lactones including zeranol

(5) Beta-agonists

(6) Compounds included in Annex IV to Council Regulation (EEC) No 2377/90 of 26 June 1990

GROUP B — **Veterinary drugs**(1) **and contaminants**

(1) Antibacterial substances, including sulphonomides, quinolones

(2) Other veterinary drugs

- (a) Anthelmintics
- (b) Anticoccidials, including nitroimidazoles
- (c) Carbamates and pyrethroids
- (d) Sedatives
- (e) Non-steroidal anti-inflammatory drugs (NSAIDs)
- (f) Other pharmacologically active substances

(3) Other substances and environmental contaminants

- (a) Organochlorine compounds including PcBs
- (b) Organophosphorus compounds
- (d) Chemical elements
- (d) Mycotoxins
- (e) Dyes
- (f) Others

Figure 1.2. Classification of drugs as unauthorized (group A) substances and authorized substances (group B) in 96/23/EC.

There is no such thing as absolute safety from chemical substances. In order to control the presence of chemicals such as veterinary drugs and pesticides in food, maximum levels based on scientific data are established. MRLs are health based levels, and are important outcomes of risk analysis approach.

The purpose of MRLs is to protect the consumer by making sure that veterinary drugs consumed in food of animal origin do not exceed the acceptable daily intake (ADI) of the substance. ADI is calculated from a no observable adverse effect level (NOAEL) which is then corrected by a safety factor for differences between animals and human differences, and then multiplied by body mass.

$$ADI=NOAEL/safety\ factor \times body\ mass$$

NOAEL is based on long term studies in laboratory animals where studies are performed with several doses of the drug and the amount of substance that shows no toxic effects is considered as NOAEL (Figure 1.3). Safety factor is conventionally 10 for difference between test animals and human and another 10 for difference between humans. The ADI is usually given in mg per kg body weight. EU MRLs are set by the European Commission followed by the opinion of Committee of Veterinary Medicinal Products (CVMP) of European Medicines Agency (EMA). Manufacturers apply for an MRL to CVMP while registering the product. MRLs for veterinary drugs are elaborated based on two dossiers of information: Safety dossier and Residues dossier. The safety dossier consists of pharmacology and toxicology studies carried out with the medicine in laboratory animals and also include calculation of ADI using NOAEL obtained with sensitive testing methods. The residues dossier consists of data concerning formation, nature, behaviour and disappearance of residues once the drug is administered to the animal [24]. Together with safety data and residue data, theoretical food consumption data is used to calculate MRL for the drug in each food commodity.

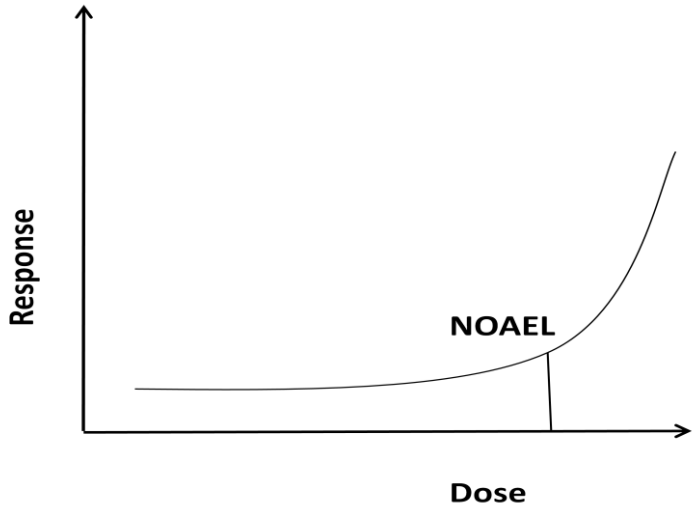


Figure 1.3. Dose response plot to establish NOAEL for a drug compound

The document listing MRLs for all pharmacologically active substances in food stuffs of animal origin is EC decision 37/2010 [25]. It has two lists of substances – Table 1 of the document listing authorized substances with full MRLs and no MRLs required and Table 2 of the document listing prohibited substances. When there is no toxicity for a particular compound, it is listed as No MRLs required. However it has to be noted the MRL for each drug is established for each food commodity and the scope of MRLs is

not exhaustive. For e.g. in honey where presence of veterinary drugs is often reported, no MRLs are established. Moreover, MRLs are established considering toxicity of one specific studied drug, without taking into account the possibility of presence of multiple drugs or synergetic effects.

For prohibited substances, according to EU Commission Decision 2002/657/EC, an analytical technique based limit called minimum required performance limit (MRPL) should be used. MRPL is the minimum concentration of analyte in a sample that has be detected and confirmed. It has to be mentioned that, for many such substances, MRPL has not been laid. Recently in 2013, EFSA panel on contaminants in the Food chain (CONTAM) recommended establishing reference points for action (RPAs), which are established on both analytical and toxicological considerations [26].

In the international scenario, Codex Alimentarius MRLs are established by JECFA for veterinary drugs and are provided in the Codex webpage [27]. It has to be noted that establishing MRLs is a time consuming and costly process and it is prudent to establish harmonized MRLs at international level.

The Commission Decision 2002/657/EC [28] implements the council directive 96/23/EC by establishing criteria and procedures for validation of analytical methods and to interpret analytical results generated by official laboratories. According to this document,

Methods are classified as screening and confirmatory methods. Screening methods are the methods that can give results < 5% error of false compliant samples at the level of interest. The suspected non compliant result of the sample obtained from the screening method has to be confirmed with a confirmatory method. Conventionally, screening methods are applied to vast number of samples and only the non compliant samples are tested again with confirmatory methods. Generally, screening methods are less expensive, less specific and rapid than confirmatory methods. However, screening methods based on mass spectrometry with minimal sample preparation are also developed in order to provide accurate and rapid test results

Confirmatory methods are the methods that can give results with < 1% false compliant rate at the level of interest for Group A substances and < 5% for group B substances. Group A substances must be monitored with liquid chromatography (LC)/gas

chromatography (GC) with mass spectrometry (MS) detection or with infra red (IR) spectrometric detection with specific requirements. Group B substance can be analyzed and confirmed by LC-UV/VIS, LC-immunogram, GC- electron capture detector, LC fluorescence, LC-full scan diode array detector, LC/GC with MS detection, LC or GC with IR spectroscopic detection.

For the validation of a quantitative confirmatory method, performance characteristics such as decision limit (CC α), detection capability (CC β), trueness/recovery, precision, selectivity/specificity, applicability, ruggedness and stability have to be determined.

During validation if the MRL is established for an analyte/matrix combination, decision limit, CCα, is calculated using precision data obtained by analyzing blank samples spiked at MRL, according to the equation CCα = MRL + 1.64 S (S = standard deviation at MRL level in terms of concentration). Detection capability, CCβ, is calculated from the CCα value according to the equation, CCβ = CCα + 1.64 S.

For banned substances, where an MRL has not been set, CCα is calculated from the calibration curve prepared by spiking blank matrices in the low concentration range. CCα is calculated as the concentration corresponding to the *y*-intercept plus 2.33 times its standard deviation. Subsequently, CCβ is calculated from the standard deviation obtained at the CCα level (CCα + 1.64 S).

According to Commission Decision 202/657/EC, the concept of identification points (IPs) is used to set up quality criteria for qualitative and quantitative methods. The basic idea of IPs is that the laboratory is allowed to use any spectrometric techniques to obtain minimum number of IPs necessary for the proper identification of the compound [29]. Confirmatory methods based on MS detection have to meet 4 identification points (IPs) for Group A (banned) substances and 3 IPs for group B substances (established MRL). In addition to that, retention time and ion ratio between two ions have to be monitored at permitted tolerances as in table 1.5 to make sure that both the ions come from the same precursor.

Table 1.5. Maximum permitted tolerances for relative ion intensities using mass spectrometric techniques in Commission Decision 2002/657/EC.

Relative intensity (% of base peak)	EI-GC-MS (relative)	CI-GC-MS, GC-MS^n LC-MS, LC-MS^n (relative)
> 50 %	± 10 %	± 20 %
> 20 % to 50 %	± 15 %	± 25 %
> 10 % to 20 %	± 20 %	± 30 %
≤ 10 %	± 50 %	± 50 %

Typically with liquid chromatography coupled with triple quadrupole mass analyzer (QqQ), one precursor and two transition products are followed to yield sufficient IPs (1+1.5+1.5). In case LC-MS/HRMS, e.g. one precursor ion and one transition product ion can be monitored (2+2.5). It has to be mentioned that the document does not lay the mass accuracy criteria for high resolution mass spectrometry (HRMS) instrumentation, which would affect the testing results to a great extent. A mass accuracy criteria of having mass measurements within 5 ppm in relative terms or an absolute value in mDa need to be established to provide confident test results.

In an international perspective, Codex draft guidelines, lay the requirements for performance characteristics of analytical methods [30]. The draft guideline adopts similar identification point criteria as in Decision 2002/657/EC. However, it includes mass measurement accuracy criteria for high resolution measurements to be within 5 ppm.

1.4. The role of analytical laboratories

Laboratories are an essential component of a food control system and become the crux of the evidence based decisions taken during a food crisis and risk analysis. Analytical data from the laboratories become the central component while setting a public policy and can have a strong influence on public opinion on scientific findings.

FAO defines the responsibilities of food safety control laboratories [15] as,

"Laboratories are responsible for analyzing food samples to detect, identify and quantify contaminants (such as pesticide residues or heavy metals) and for analyzing specimens from humans and foods implicated in food-borne illness outbreaks to identify

causes and sources. They also provide support for food law enforcement. The scientific information produced by food control laboratories also informs and supports policy and decision making processes related to food safety and quality, for instance to design surveillance and monitoring programs that target priority hazards, consumer complaints, disease outbreaks, etc and other emerging food safety and quality issues".

In the EU, European Commission regulation 882/2004 on official controls [31] established a network of laboratories comprising, community reference laboratories (CRLs), National reference laboratories (NRLs) and official control laboratories (OCLs). CRLs are at the top level and their main responsibilities are organizing comparative tests among NRLs, informing about the advances in analytical methods to NRLs and provide technical assistance to the EU. The list of community reference laboratories for veterinary drug residues and chemical contaminants in food of animal origin and feed, adapted from [32] is provided in table 1.6.

Table 1.6. List of community reference laboratories for veterinary drugs and chemical contaminants in feed and foods of animal origin reproduced with permission from Companyó et.al.[32].

Laboratory	Groups of substances
Rijksinstituut voor Volksgezondheid en Milieu (RIVM) 3720 BA Bilthoven. The Netherlands	Group A 1, 2, 3, 4, Group B 2(d) and Group B 3(d). Annex I. Directive 96/23/EC
Laboratoire d'études et de recherches sur les médicaments vétérinaires et les désinfectants AFSSA — site de Fougères. BP 90203. France	Group A 6, Group B 1 and B 3(e). Annex I. Directive 96/23/EC. Carbadox and olaquindox
Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL) 12277 Berlin. Germany	Group A 5 and Group B 2(a), (b), (e). Annex I. Directive 96/23/EC
Instituto Superiore di Sanità 00161 Roma. Italy	Group B 3(c). Annex I. Directive 96/23/EC
Danmarks Fodevareforskning (DFVF) 1790 Kovenhavn V. Denmark	Pesticides in feed
Chemisches und Veterinäruntersuchngsamt (CVUA) 79123 Freiburg. Germany	Pesticides in food of animal origin
The Joint Research Center of the European Commission Geel. Belgium	Heavy metals in feed and food
The Joint Research Center of the European Commission Geel. Belgium	Polycyclic aromatic hydrocarbons in feed and food
Chemisches und Veterinäruntersuchngsamt (CVUA) 79123 Freiburg. Germany	Dioxins and PCBs in feed and food

NRLs are responsible to organize interlaboratory exercises between official control laboratories and facilitate communications from CRL to all official laboratories. Decision 2006/130 establishes the list of NRLs for detection of residues [33] . An example network of laboratory in Spain is shown in figure 1.4.

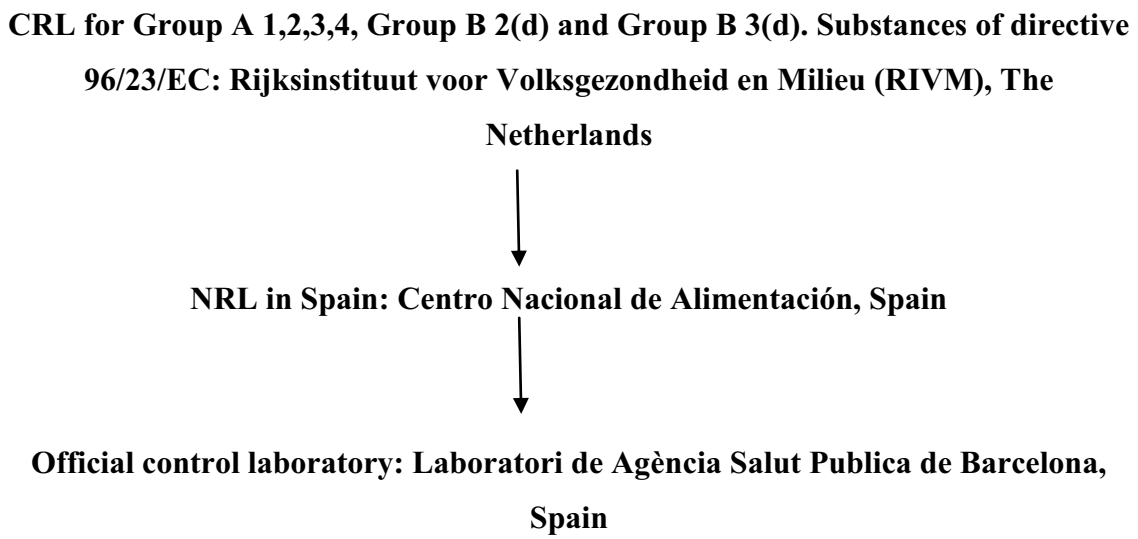


Figure 1.4. An example network of laboratories for analyzing veterinary drug residues

An official control laboratory can be publicly or privately owned, and are appointed by national food safety authorities for official control purposes and should meet specific requirements [34].

- accreditation by a national accreditation body (for e.g. ENAC in Spain) in accordance with requirements of ISO/IEC 17025:2005 [35]
- satisfactory performance in external proficiency testing schemes.
- the use of validated methods
- the employment of suitably qualified persons to carry out the analysis.

1.4.1. Method validation

Analytical method validation is a process that ensures that the method is fit for the specified purpose. The ISO definition of validation is as follows : 'Confirmation by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled' [36]. Method validation is at first level in the quality assurance in a laboratory as illustrated in the figure 1.5. The purpose of an analytical method is to deliver a quantitative result with an acceptable uncertainty level. Validation in other words mean measuring uncertainty and in practice, validation is done by evaluating method performance characteristics such as precision, trueness, selectivity/specificity, linearity, linearity range, recovery, limit of detection, limit of quantitation, sensitivity, ruggedness and applicability. For methods following 2002/657/EC, decision limits and detection capabilities are also calculated.

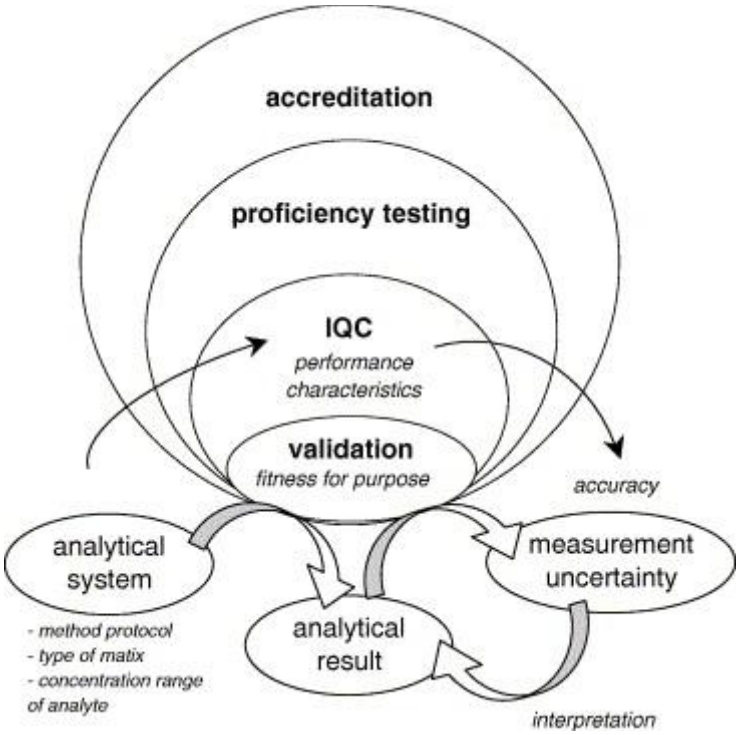


Figure 1.5. Schematic of quality management in a laboratory, reproduced with permission from Taverniers et.al. [37]

1.4.2. Quality management (reference materials and interlaboratory comparison)

Apart from method validation, laboratory has to undertake a series of activities to ensure the quality of results. The activities include internal quality control procedures by using reference materials, certified reference materials, control charts, etc; participation in proficiency testing schemes and accreditation to ISO/IEC 17025:2005 (Figure 1.5).

Reference materials can be virtually any material used as a basis for reference, and could include laboratory reagents of known purity, industrial chemicals, or other artefacts. The property or analyte of interest needs to be stable and homogenous but the material does not need to have the high degree of characterization, traceability and certification more properly associated with certified reference materials.

The characterization of the parameter of interest in a certified reference material is generally more strictly controlled than for a reference material, and in addition the characterized value is certified with a stated uncertainty by a recognized institution. Characterization is normally done using several different methods, so that as far as possible, any bias in the characterization is reduced or even eliminated.

The accredited laboratories are obliged to participate in interlaboratory exercises regularly, in order to assure the quality of the results provided by the laboratory. Some organizations that are involved in organizing proficiency testing schemes for veterinary drugs are Food Analysis Performance Scheme (FAPAS), French Agency for Food, Environmental and Occupational Health and Safety (AFSSA) and Progetto Trieste.

The organizing entity send samples with accurately measured concentration of analyte to the participating laboratories and the laboratories have to provide results within a mentioned date. After receiving the results, the organizer assigns Z score for each laboratory, which is calculated as follows

$$z = \frac{x - \mu}{\sigma}$$

Where, x= result obtained by the participating laboratory

μ= average of all the results from participating laboratories except those considered as outliers

σ = standard deviation of the results accepted

A z score between ±2 is considered satisfactory, a score between ±2 and ±3 is considered questionable and a score above that is incorrect.

When the laboratories do not attain satisfactory results, corrective action has to be taken and documented.

1.4.3. Laboratory accreditation – ISO/IEC 17025:2005

According to Regulation EC 882/2004 all the laboratories in Europe involved in food safety controls must be accredited by a national accreditation body (for e.g. ENAC in Spain) as per ISO/IEC 17025:2005 [35] The standard is specific to the competence of testing and calibration laboratories in all the fields including chemical, biological, environmental, materials and physical testing. It has two parts – management requirements to ensure the operation and effectiveness of quality management system, and technical requirements to ensure the reliability of the tests and calibrations performed in the laboratory. Accreditation according to ISO/IEC 17025 was developed in the concept of “tested once, accepted everywhere”.

2. Analytical methodologies

Analytical methodologies typically consist of a sample preparation (extraction and/or clean up) step, followed by separation and detection of analytes. In the veterinary drug residue analysis, liquid chromatography is the preferred separation technique and mass spectrometry is the preferred detection technique.

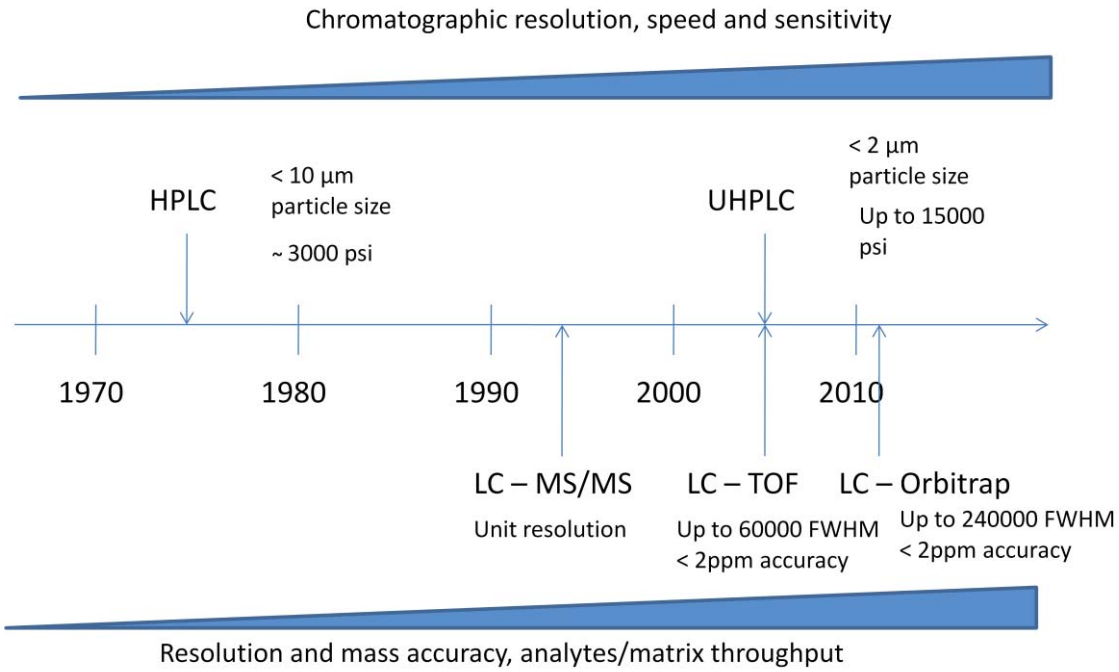


Figure 2.1. Timeline of development of liquid chromatography and mass spectrometry.

In residue analysis, in the liquid chromatography side, high performance liquid chromatography (HPLC) was first used in the mid 1970s. Later in the mid 2000s, ultra high performance liquid chromatography (UHPLC) was introduced with sub 2 µm particle sizes and backpressures reaching up to 15000 psi (Figure 2.1). Such low particle sizes and high backpressures have improved separation, reduced chromatographic run time drastically and gives good limit of detection (LODs) due to sharp and tall peaks. In the mass spectrometry side, low resolution triple quadrupole mass analyzers for residue analysis were introduced in the mid 1990s and were widely accepted due to their robustness and ease to use. Later, high resolution mass analyzers such as TOF and Orbitrap were introduced which show promising capabilities to analyze numerous analytes in the same method and provide accurate mass measurements.

To use mass spectrometers for confirmatory methods, the laboratories should comply the identification point criteria according to EU Commission Decision 2002/657/EC or CAC 2012. In case of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), retention time (RT), two MRM ions – quantifier and qualifier, and an ion ratio between these two ions have to be monitored. In case of HRMS, two ions at high resolution have to be monitored at mass measurement accuracy less than 5 ppm and at resolution higher than 20,000 FWHM. However, identification point and ion ratio criteria has been criticized to be based on expert judgement based rather than to be based on scientific evidence.

2.1. Sample preparation

Sample preparation is an important part of the analytical method, which is essential to achieve reliable results and maintain instrument performance. Sample preparation is the process of extracting analytes from a sample and usually followed by a clean-up step to further eliminate matrix interferants and isolate the analytes. In targeted analysis, where known analytes are analyzed, effective removal of matrix and selective preconcentration of analyte is the aim of an efficient sample preparation.

The most commonly used extraction techniques are solid liquid extraction or liquid liquid extraction using organic/aqueous solvents or buffers depending on the polarity and pKa of the analytes. Acetonitrile is a preferred extraction solvent as it achieves good recovery of analytes and extracts low levels of matrix. Methanol and ethanol are other widely used solvents [38]. The instrument based extraction techniques such as microwave assisted extraction (MAE), supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE) are also used. The instrumental technique is advantageous for automation and selective isolation of residues.

Solid phase extraction (SPE) is the most commonly used clean-up technique, where sorbents with silica or polymeric supports with various functional groups are used. Depending on the functional groups and solvents used, SPE can be applied as normal phase, reversed phase, ion exchange and mixed mode phase. Apart from SPE, dispersive SPE (QuEChERS), molecular imprinted polymers (MIPs), immuno affinity chromatography are also used as clean up techniques. Automated offline/ online SPE,

turbulent flow chromatography, are some of the high throughput techniques used for purification [38].

2.2. Liquid chromatography

For confirmation of veterinary drug residues in food, liquid chromatography coupled to mass spectrometry is the preferred technique due to selectivity of mass spectrometer related to other detection systems such as UV-VIS or fluorescence detection [39].

Liquid chromatography is the preferred separation technique for veterinary drugs because of its possibility to apply for various chemical families that are usually polar, non volatile and sometimes heat sensitive [40]. The chromatographic process involve injection of a sample extract into a column, which is conditioned with a solvent (mobile phase) flow. The separation takes place within the column and the sample components are eluted to the detector. The nature of separation depends largely on kind of stationary phase (functional group), porous or core shell, particle size (1.5 to 5 μm), mobile phase composition, and temperature and flow rate.

The most common type of stationary phases used is reverse phase chromatography (RPC). In RPC, stationary phase is non polar (e.g. C18) and the mobile phase is a polar mixture of water and organic solvent. Hydrophilic interaction chromatography (HILIC) is another chromatographic technique that is gaining attention for analyzing highly polar compounds. HILIC is an alternative to normal phase chromatography (NPC), where stationary phase is polar (e.g. silica) and mobile phase is a mixture of water and organic solvent, but water is the strong eluent [41].

2.3. Atmospheric Pressure Interfaces (API)

For confirmation of veterinary drug residues in food, liquid chromatography coupled to mass spectrometry is the preferred technique due to selectivity of mass spectrometer related to other detection systems such as UV-VIS or fluorescence detection [39]. The development of mass spectrometer interface is an important factor for wide acceptance of MS as detection technique with LC. MS can detect ions in the gaseous phase. To use MS as a detector, the LC fluent has to be evaporated and generate ions, which is the function of interface. Soft ionization techniques ionize with minimal degradation of analytes and the most commonly used techniques are i) Electrospray ionization (ESI) and ii) atmospheric pressure chemical ionization (APCI). Recently

atmospheric pressure photoionization (APPI) is gaining more attention due to less matrix effects. Each source has different ion suppression mechanism and can provide interesting advantages for residue analysis, where matrix ion suppression is a serious problem [42]. The softness of the ionization technique is the following order ESI>APCI>APPI. The applicability of the APIs is shown in figure 2.2. For small molecule (<1000Da) identification, APCI and APPI can be good ionization sources.

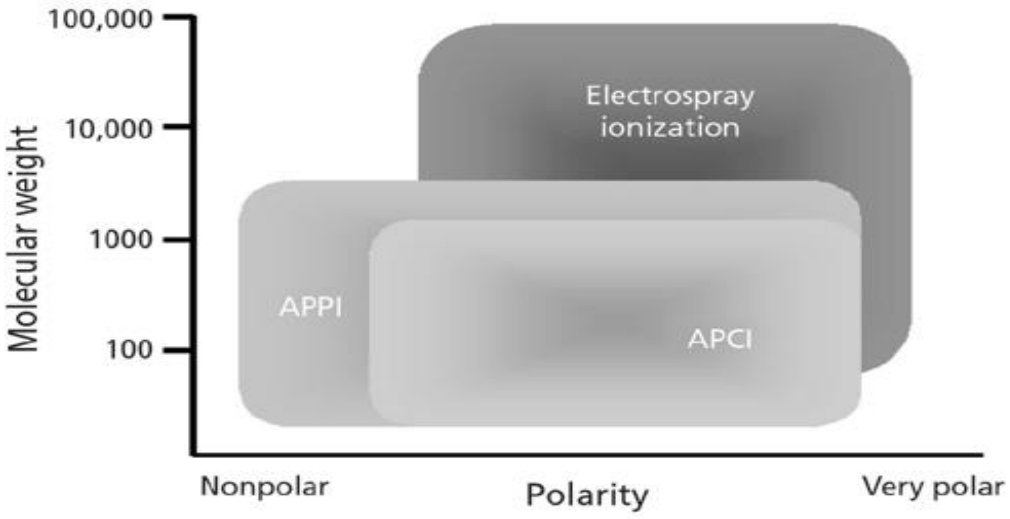


Figure 2.2. Atmospheric pressure interfaces and their applicability to compounds of varying polarity and molecular weight adapted from Syage et.al. [43].

Electrospray ionisation is the most widely used ionization technique, with high ionization efficiency over a dynamic mass range of compounds. Mass spectrometers are continuously evolving to increase the ion transmission of interfaces and mass spectrometers to take advantages of high ionization efficiency of ESI. In ESI, the mobile phase is sprayed into a heated interface, where the solvent is evaporated and leave analyte ions in the gas phase. An electric potential (e.g. 3-5kv) is applied on the nebulizer spray tip (capillary) to ionize the analyte molecules. There are two major theories that explain the final production of gas phase analyte ions. The Ion Evaporation model (IEM) and the Charge Residue Model (CRM). IEM (Figure 2.3) suggests that when the droplet shrinks to certain radius, the field strength at the surface of the droplet becomes large enough to expel the solvated ion.[44]

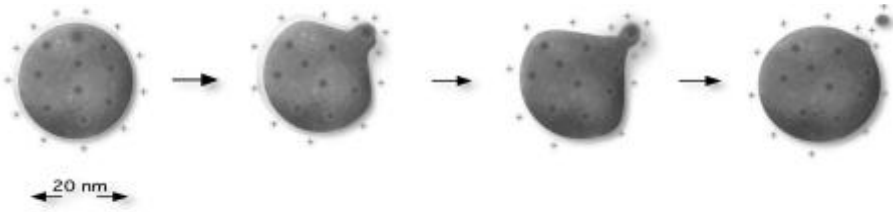


Figure 2.3. Schematic of ion evaporation model reproduced with permission from Wilm [44]

A charge residue model (Figure 2.4) assumes that electrospray droplets undergo evaporation and fission cycles until smaller droplets containing one analyte ion are formed. The molecule is released as ion by solvent evaporation and declustering.

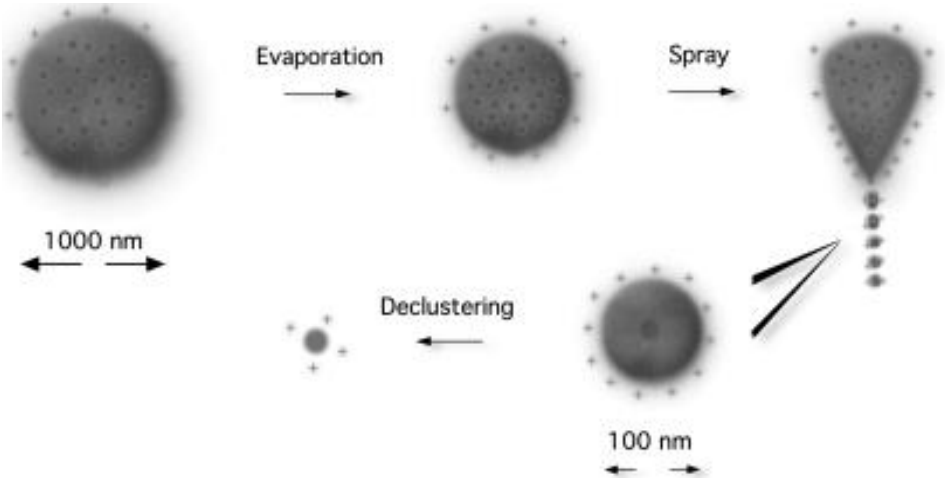


Figure 2.4. Schematic of charge residue model reproduced with permission from Wilm [44]

Although ESI works best at 1-20 µl/min, use of nebulizer gas such as N_2 allows higher liquid flow from LC. In positive mode typical ions formed are $[M+H]^+$, $[M+Na]^+$ and also $[2M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ can be observed at high concentration of analytes. In negative ionization $[M-H]^-$ is the typical ion and $[M+Cl]^-$ can also be observed.

APCI is another atmospheric pressure ionization interface. It vaporizes the mobile phase first and uses a corona discharge to add charge to analyte in gas phase. In APCI no spray voltage is applied. APCI can be used for some non polar compounds which do not ionize well with ESI, but analytes can be degraded more than ESI. APCI requires high liquid flow (200- 1000 µl/min) for effective vaporization.

In APPI, an ultra violet (UV) lamp is used to generate photons which ionize the analyte. Energy of UV photons and ionization energies of analytes dictate the initiation of APPI process [45]. APPI could be useful for compounds that are not amenable with ESI and APCI, especially non polar compounds which are analyzed with GC-MS alone. APPI seems to be less susceptible to matrix suppression and buffer created chemical noise [46]. Dopant such as toluene or acetone can be used to effectively photoionize and act as charge carrier for ionizing trace level of analytes.

2.3. Mass spectrometry

Mass spectrometry has a dynamic history with a continually progressing technology that has made significant advances into different fields such as environmental analysis, food safety, drug discovery, clinical and forensic analysis, protein characterization, and disease diagnosis. Moreover, MS was generally accepted for identification and quantification of compounds since 1960s with the coupling of GC-MS, LC - MS and the development of MS/MS [47].

In the field of mass spectrometry, various terminologies are being used which leads to inconsistent usage, especially while using accurate mass measurements. The definitions of nominal mass, accurate mass, exact mass and monoisotopic mass are summarized in table 2.1 -The terminologies to be used are discussed in detail by different authors [48, 49]. Moreover, in order to standardize the definitions, International Union of Pure and Applied Chemistry (IUPAC) has compiled a list of standards and definitions [50]. A guidance for terminology and treatment of data has also been described in detail by Brenton [51].

Table 2.1. Terminologies for accurate mass measurements.

Nominal mass	The mass of an ion or molecule calculated using the mass of the most abundant isotope of each element rounded to the nearest integer value and equivalent to the sum of the mass numbers of all constituent atoms
Accurate mass	The experimentally determined mass of an ion measured to an appropriate degree of accuracy and precision used to determine, or limit the possibilities for, the elemental formula of the ion
Exact mass	The calculated mass of an ion whose elemental formula, isotopic composition, charge state are known i.e. the theoretical mass. The IUPAC

	definition constricts the definition to using one isotope of each atom involved, usually the lightest isotope, but generalizes the definition to cover an ion or neutral molecule. The charge state is relevant as the mass of the electron (0.00055Da) or multiple charges, may not be negligible in the context of mass measurement
Monoisotopic mass	The exact mass calculated using the mass of the most abundant isotope of each element.

2.3.1. Mass measurement accuracy

The difference between the measured value (accurate mass) and the true value (exact mass) is the "accuracy" of the "accurate mass measurement" and it is suggested that the term "mass measurement accuracy" should be used to denote this difference [51].

The mass measurement error (or accuracy)

$\Delta m_i = (m_i - m_a)$ in Da

$= (m_i - m_a) \times 10^3$ in mDa

$= \frac{m_i - m_a}{m_a} \times 10^6$ in parts per million (ppm)

Where m_i is the measured accurate mass in Da and m_a is the calculated exact mass in Da.

It has to be noted that mass accuracy in ppm is a relative value. With increase in the mass measured, the mass accuracy value decreases (improves).

2.3.2. Mass resolving power and resolution

Resolving power is the capacity of a mass spectrometer to separate ions. It is defined either as Full Width at Half Maximum (FWHM) of the peak or as 10% height of each peak. Resolution is the measure of separation of two mass spectral peaks. The difference between resolution and resolving power is depicted in figure 2.5.

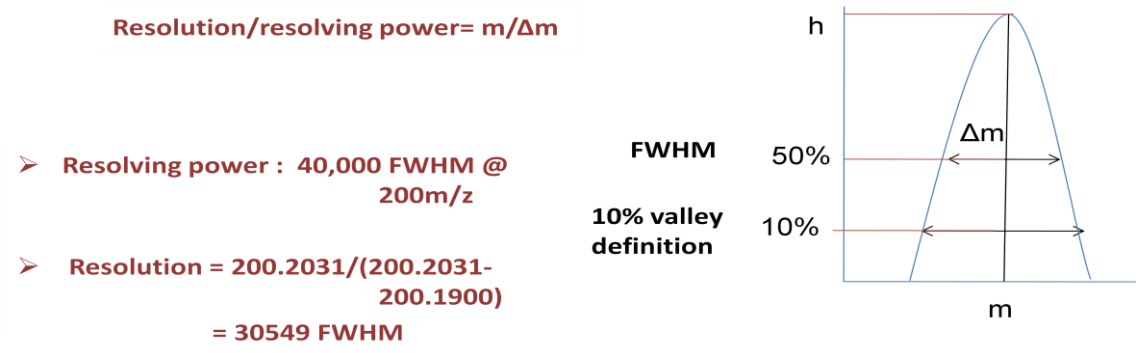


Figure 2.5. Resolution and resolving power in high resolution mass spectrometry

2.3.3. Mass analyzers

2.3.3.1. Triple quadrupole mass analyzer

The triple quadrupole mass analyzer (QqQ) is the commonly used hybrid mass spectrometer in food testing laboratories for quantitation and confirmation of veterinary drugs and also other organic contaminants. However, quadrupole is a low unit resolution mass analyzer. QqQ has three quadrupoles in tandem. Q symbolizes the quadrupole mass analyzer and q represent radio frequency (RF) only non mass resolving quadrupole, which acts as a collision cell using a collision gas

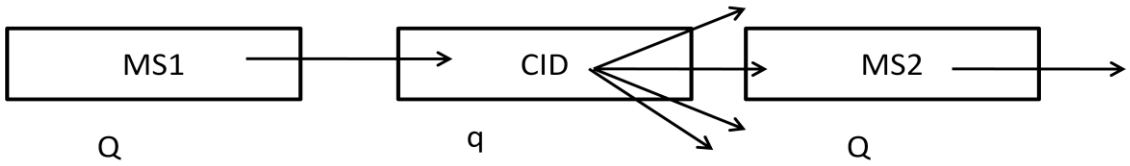


Figure 2.6. Schematic of a SRM working mode in triple quadrupole mass spectrometer.

The instrument can scan in several ways and the most important scanning modes are i) product ion scan ii) precursor ion scan iii) neutral loss scan iv) single reaction monitoring (SRM) or multiple reaction monitoring (MRM)

The SRM/MRM is the most used scan mode for the confirmation of presence of veterinary drugs to get two transition product ions and satisfy the identification point criteria.The SRM mode involves selecting an ion with chosen m/z ratio with first quadrupole. The selected ion collides in the second quadrupole and forms product ions

(Figure 2.6). The reaction product is then selected in the second mass analyzer. When more than 1 reaction products of a precursor is monitored, then it is called as MRM (usually 2)

2.3.3.2. Time of Flight (TOF)

The first Time of Flight (TOF) analyzer was constructed in 1946 by Stephens [52]. The principle of TOF is as follows: ions of different m/z are allowed to fly freely along a field free path of known length. The lighter ions will reach the detector earlier than the heavy ones. The mass resolving power depends on time of flight. Increasing the time of flight increases the resolving power.

Many variants of TOF analyzers have been developed – reflectron TOF, orthogonally accelerated TOF to improve resolving power. Currently TOF analyzer typically can achieve mass resolution between 1000 and 50,000 FWHM at 1000 m/z and mass accuracy between 1 and 5 ppm [51]. However, it has to be noted that it is typical to do internal calibration of mass spectrometer to achieve good mass accuracy. The drift in mass measurements can occur due to environmental factors such as room temperature and vacuum. The hybrid instrument such as quadrupole TOF (Q/TOF) with fragmentation cell is advantageous to achieve fragments which are important for confirmation of identity of chemical substances in fields such as food safety testing. A recent book volume on Comprehensive analytical chemistry series [53] review various TOF applications in food and environmental testing.

2.3.3.3. Orbitrap

The Orbitrap mass analyzer is a new addition to HRMS, which is the only mass analyzer invented after TOF. The Orbitrap was invented in 1999 [54] and first commercialized in 2005.

Principle: The Orbitrap consist of a spindle shaped central electrode and an outer electrode split in half by an insulated ceramic ring. Ions injected from C shaped ion trap (C trap) move in spiral around the electrode and create an axial field gradient. The frequency of harmonic axial oscillation (w_z) is determined by image current detection induced by moving ions through a differential amplifier connected to halves of the outer electrode. The image current signal is then transformed into a frequency domain signal by Fourier transform, resulting in an accurate reading of their m/z.[45].

The mass resolving power is inversely proportional to square root of (q/m)

$R = 1/2\Delta w_z (kq/m)^{1/2}$

where w_z - frequency of axial oscillations

m – ionic mass
q – ionic charge
k – field curvature

More the transient time, more the resolving power. Variants of Orbitrap include high field Orbitrap and ultrahigh field Orbitrap which are smaller versions of Orbitrap, which can reach resolving powers of up to 450,000 FWHM at 200 m/z. Mass accuracies of up to 1-2 ppm can be achieved and sub ppm accuracies can be achieved by post calibration of acquired masses [51]. The characteristics of different Orbitrap based instruments are summarized in table 2.2. Orbitrap requires ultrahigh vacuum for long transient time. Ion injection through C trap is an important one to make the m/z analysis with Orbitrap practical. C trap is a bent RF only quadrupole which accumulate, store and cool the ions (collisional damping) by low pressure nitrogen gas prior to ion injection.

Hybrid mass spectrometers such as quadrupole ion trap (QIT)/Orbitrap, Q/Orbitrap and also QIT/TOF and Q/TOF, provides the possibility of acquiring precursor isolated MS/MS and moreover smart data dependent acquisitions [55]. The precursor ion selection in Orbitrap instruments avoids post interface suppression due to matrix ions in the C trap [56, 57].

3. Trends in analytical methodologies

In food safety laboratories, conventionally targeted methods with low resolution QqQ are used mainly so far due to their robustness and sensitivity. Multianalyte targeted methods with QqQ, due to the dwell time limitations, can analyze typically up to 150 compounds in the same method. The last generation QqQ analyzers can analyze more number of compounds due to faster dwell times. However, apart from targeted analytes (e.g. veterinary drugs) belonging to few chemical families, there is possibility of presence of other contaminants such as pesticides, food contact materials, natural toxins, etc in the same sample. This inherently demands the change in testing methods from providing binary (residue present/ not present) results to comprehensive results (sample safe/ unsafe). This can be attained by comprehensive analysis of both targeted

Table 2.2. Performance characteristics of various Orbitrap instruments

	Orbitrap based instruments					
	Exactive	**Q Exactive**	**LTQ Orbitrap XL**	**Orbitrap Velos Pro**	**Orbitrap Elite**	**Orbitrap Fusion Tribrid**
Hybrid Combination	Orbitrap	Quadrupole+Orbitrap	Linear Ion trap + Orbitrap	Linear Ion trap+Orbitrap	Linear Ion trap+High Field Orbitrap	Linear ion trap, quadrupole and ultra high field Orbitrap
Maximum resolving power (FWHM)	100,000 @ m/z 200	140,000 @ m/z 200	100,000 @ m/z 400	100,000 @ m/z 400	240,000 @ m/z 400	450,000 @ m/z 200
Accuracy	<5ppm (external),<2ppm (internal calibration)	<5ppm (external),<1ppm (internal calibration)	<3ppm (external),<1ppm (internal calibration)	<3ppm (external),<1ppm (internal calibration)	<3ppm (external),<1ppm (internal calibration)	<3ppm (external),<1ppm (internal calibration)
Scan Rate	up to 10 Hz	up to 12 Hz	-	-	-	-
Polarity Switching	one full cycle in < 1 sec	one full cycle in < 1 sec	-	-	-	-
Dynamic Range	>4000	>5000:1	>4000	>5000:1	>5000:1	>5000:1

and non targeted analytes in the samples preferably at low concentration levels. Recently, high resolution mass spectrometry has evolved rapidly and offer impressive performance characteristics opening up new possibilities in food safety testing. With the advances in HRMS instrumentation (ultra high resolution and accurate mass) and the rising need for analysis of multi residue and non targeted analysis in food safety, methodologies for comprehensive targeted and non targeted analysis are being developed. Comprehensive analysis involves generally a universal generic extraction method, followed by a full scan analysis with high resolution and accuracy for targeted and non targeted analysis.

The extraction of multifamily analytes generally involves generic and non selective methods such as QuEChERS [58-60] or liquid-liquid extraction of sample with water/organic solvent mixtures [61, 62]. Although this approach can extract most of the analytes, it would also extract a lot of matrix components, which is not the case in selective sample preparation approaches like SPE. In this case, the high resolution and mass accuracy instruments would be able to resolve the matrix interferences. In food and feed testing, resolution greater than 50,000 FWHM is shown to be necessary to resolve isobaric interferences and accurate mass assignment of the peaks [63]. However, ion suppression of analytes can occur in electrospray ionization (ESI) due to coeluting matrix interferences [64]. Moreover, a study made by Croley et.al [65]., with six coeluting isobaric compounds showed a reproducible mass shift in Orbitrap due to isobaric coeluting compound leading to increase in mass error. Also post interface suppression in a single stage Orbitrap due to matrix interferences was reported [57].Such mass shift errors and ion suppression (interface and post interface) shows the need for a good chromatographic separation even when combined with high resolution and mass accuracy.

3.1. Multifamily targeted methods

Targeted methods generally involve previous characterization of compounds with the use of standards. In this part, selected multifamily targeted methods (summarized in table 3.1) with LC-HRMS systems (TOF and Orbitrap) in food testing, covering more than 100 analytes are discussed. In the table it can be seen that Orbitrap based methods are able to use a mass accuracy less than 5 ppm and TOF based methods have higher mass measurement error. Full scan HRMS instruments are typically used for screening purposes rather than confirmation due to the lack of required sensitivity or lack of

designated fragmentation cell possibility of the equipment. Although in- source fragmentation can be used, it is not a successful approach to produce fragments for all the analytes. In a quantitative method [66], developed with LC-TOF/MS, detection and quantitation can be carried out with retention time and exact mass, but confirmation requires the analysis of suspected sample with LC-MS/MS to produce fragments. This is because, the accurate mass of precursor ion and isotopic pattern can only confirm the elemental composition of the analyte. In order to confirm the structure of the compound and thus confirm the presence of the compound, fragments need to be monitored. The reliability of compound confirmation increases when the fragments are monitored in addition to precursor ion. An assessment study by Stoev et. al.[67] shows that, to achieve selectivity of a low resolution (unit mass) selected reaction monitoring (SRM) of a triple quadrupole analyzer, relying solely on a precursor ion in HRMS would require a resolution of above 1 million.

A comparative study [68] with 240 analytes in three different matrices of LC-MS/MS and HRMS shown that the quantitative performance such as linearity and sensitivity of a single stage Orbitrap system is similar to that of a LC-MS/MS system. Similar studies comparing LC-MS/MS and LC-QqTOF shows that QqTOF is less sensitive [69] and poorly repeatable [70] than LC-MS/MS. Moreover validation of multiresidue methods is difficult due to complexity in preparation of multiple analyte standards having varying MRLs and physiochemical properties [60, 71]

3.2. Identification in non targeted analysis

Considering that most of the compounds in the sample are ionized in the source and detected by the mass spectrometer at high resolution and accurate mass in Full MS, the non targeted analysis involve assigning an elemental composition, structure and thereby identifying the compound. It has to be noted that the mass of the electron (0.000549 u) has to be included in the exact mass calculation. Omitting that can result in an error of up to 5 ppm depending on the mass (Figure 3.1). Also, in order to improve the accuracy of the measured masses, post acquisition mass recalibration using internal standards can be carried out to avoid systematic errors [55]. Recently Stravs et. al. [72], has been recalibrating the MS/MS spectra to attain sub-ppm mass accuracies for the fragments.

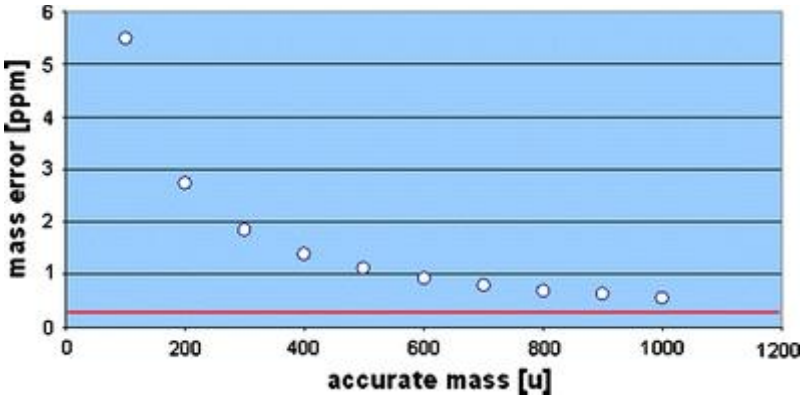


Figure 3.1. Increasing mass error with reducing accurate mass, reproduced with permission from Kind et.al.[55].

Kind and Fiehn [73] developed seven golden rules to get elemental compositions from the measured mass spectrum. Except the seventh rule, which is to use the presence of trimethylsilylated compounds with GC-MS, other rules can be used in LC-MS. These rules are useful to narrow the number of probable elemental formulas and have been successfully tested in a wide range of compounds.

From the accurate mass, the elemental formula can be searched for a compound either in an in-house database or a spectraless databases such as Chemspider or Chemical Abstract Service (CAS) registry with SciFinder user interface. CAS registry has 54 million entries which can be searched with molecular formulae or average molecular weights [82]. Chemspider is a free to use database and contains 26 million entries [83]. It provides the possibility to search molecular formula and monoisotopic mass with a specified error as obtained from the mass spectrum. In Chemspider, it can be seen that most of the chemicals fall within the range of 200-600 Da (Figure 3.2). So, masses outside this range will have less number of hits in the search. There are very few spectral databases for LC-MS due to the instrument dependent ionization and fragmentation patterns. However, an extensive in-house database of accurate mass spectra for 2500 toxic compounds which includes drugs, pesticides, alkaloids and other toxic chemicals and metabolites was created with a LC-QTOF instrument [84].

Table 3.1. List of selected multi analyte, multi family analytical methods based on HRMS.

		No. of analytes	Classes	Matrix	Extraction method	Chromatography	Detection	Method Limits	Criteria for confirmation	Measurement mass accuracy	Reference and Year
1	Screening method	258	pesticides, mycotoxins, plant toxins and veterinary drugs	Feed, maize, meat, milk, egg and honey	Solid liquid extraction	UPLC waters, 1.7 µm, 100x2.1mm ID	MS/MS and TOF MS	LOD < 0.01 to 0.05 mg/kg for most analytes	accurate mass and RT	less than 5 ppm	Mol et.al., 2008 [62]
2	Confirmatory method	250	Pesticides	three different matrices	NA	NA	Exactive	NA	NA	NA	Kaufmann et.al., 2012 [68]
3	Screening method	150	veterinary drugs	raw milk	Solid liquid extraction	UPLC waters, 1.7 µm, 100x2.1mm ID	TOF MS	LOD- 0.5 to 25 µg/l	accurate mass and isotopic pattern	0,02 Da mass window	Ortelli et.al., 2009[74]
4	Screening method	87	banned veterinary drugs	urine	Quechers	UPLC,	Exactive	CCβ - 0.2-20 µg/l	retention time and accurate mass of precursor, isotopic pattern	5 ppm	Leon et.al., 2012[75]
5	Quantitative	100	veterinary drugs	muscle, kidney, liver, fish and honey	Extraction with ACN, EDTA, amm sulfate, SPE, Evaporation	Kinetex C18, 150 *2.1 mm, 2.6 µm	Exactive	NA	retention time, accurate mass of precursors	10 ppm	Kaufmann et.al., 2011[76]

6	Quantitative	100	veterinary drugs	muscle kidney, liver	Extraction with different solution, SPE with oasis hlb, Evaporation	acquity uplceHSS T3 column	TOF	CC α - 1-5 µg/kg	quantitation with TOF and confirmation with MS/MS	60 ppm	Kaufmann et.al., 2008[66]
7	Screening method	127	veterinary drugs	bovine muscle	Quechers	acquity uplceHSS T3 column - 100 *2.1, 8µm particle size	LC -MS/MS	NA	NA	NA	Asteggiante et.al., 2012 [60]
7	screening method	63	veterinary drugs	bovine and swine muscles	extraction with SPE, C18, bond elut	rp18e, purospher column thermo	LTQ Orbitrap	LOD - 1 to 300 µg/kg.	accurate mass and retention time	5 ppm	Hurtaud et. al., 2011 [77]
8	screening method	116	Pesticides	various food matrices	Turboflow	hypersil gold 2,1*100mm,1,9µm	Exactive	LOQ- 1 to 50 ng/g	accurate mass and retention time	5 ppm	Shi et. al., 2011[78]
9	screening method	150	plant toxins	various food and feed matrices - silage, honey, pig feed, food supplement	Quechers	atlantis T3 LC 100*3 mm, 3µm	Exactive	LOD - 10 to 200 µg/kg	accurate mass and retention time	5 ppm	Mol et. al., 2011[58]
10	Screening method	118	plant alkaloids, carbamates and organophosphate pesticides, and veterinary drugs	liver, muscle, milk, silage	Quechers	Hypersil gold AQ 2,1*50mm, 1,9 µm	Exactive	NA	accurate mass and retention time	within 2ppm	Filigenzi et. al., 2011[59]

11	qualitative and quantitative determination	350	veterinary drugs and pesticides	honey	water and acetonitrile, dissolvation	Hypersil gold AQ 2,1*100mm, 1,7 µm	Exactive	LOD - 1 to 50 µg/kg	accurate mass, isotopic pattern and retention time	less than 5 ppm	Gomes et. al., 2012[61]
13	screening and confirmation	29	veterinary drugs	milk and powdered milk infant formula	Quechers	Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm	Exactive, QTOF, MS/MS	LOD- 0.2 to 5 µg/kg	accurate mass, isotopic pattern, retention time and fragments (for confirmation)	10 ppm for QTOF and 5 PPM for exactive	Gonzalez et.al., 2011[79]
12	confirmatory method	138	pesticides	fruit and vegetable based infant food	Quechers	Acquity UPLC BEH C18 column, 100 mm × 2.1 mm, i.d., 1.7 µm particle size	QTOF	LOD up to 1 µg/kg	accurate mass, product ions, retention time	between 2 and 10 ppm	Wang et.al., 2009 [70]
13	screening method	100	veterinary drugs	egg, fish and meat	SPE	Waters Acquity UPLC BEH C18 analytical column 100 mm × 2.1 mm and 1.7 µm	TOF	NA	accurate mass, retention time,	below 10 ppm	Peters et.al., 2009[80]
14	confirmatory method	100	pesticides	Fruit based soft drinks	SPE	C8 analytical column of 150 mm × 4.6 mm and 5 µm particle size (Zorbax Eclipse XDB-C8)	TOF MS	LOD – 0.006 to 0.03 µg/kg	accurate mass and retention time	2 ppm	Garcia et.al., 2007[81]

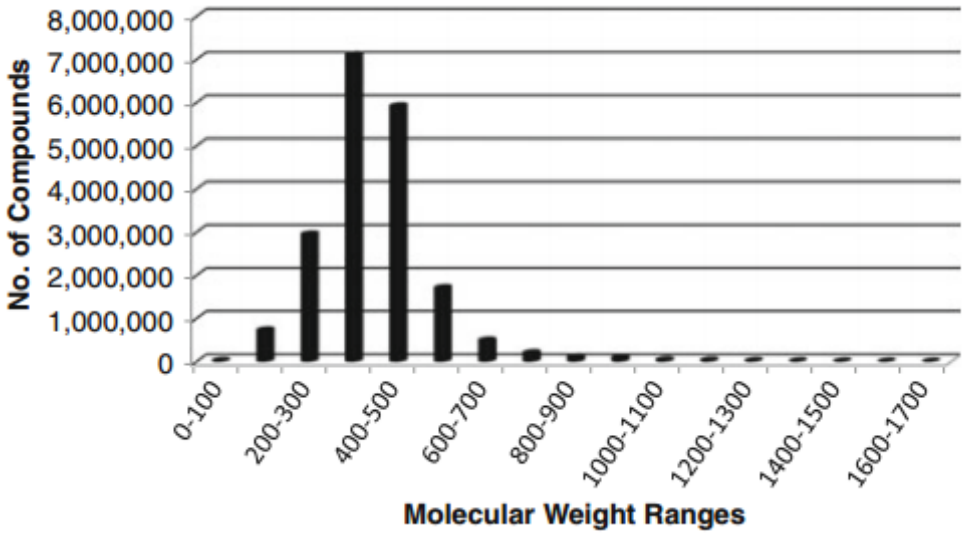


Figure 3.2. Number of Chemspider entries versus molecular weight ranges, reproduced with permission from Little et.al.[83].

Apart from the precursor mass and the isotopic pattern obtained from the Full MS, tandem hybrid instruments can provide precursor isolated fragments which can aid in identification of unknown peaks. MetFrag, an open source web application for insilico fragmentation creates fragments based on set rules for all the hits in the list and compares with the experimental mass spectra, thereby providing a score for each analyte in the list [85]. MZmine 2 is another open source software which predicts chemical formula based on heuristic rules, isotopic pattern and also MS/MS fragmentation [86].

3.3. Comprehensive analysis of targeted and non targeted compounds

Targeted methods are usually developed for the chemicals/contaminants that pose health risks and that are included in the control programs of the food safety authorities. This targeted approach has a big disadvantage in food safety perspective as they are blind to many other unexpected or not controlled chemicals in the food chain. Sometimes targeted approaches can fail seriously in cases like unexpected melamine contamination in food, which affected a large population recently [87]. In order to strengthen the tools to ensure food safety, non targeted approaches are quite important. Full MS HRMS provide the possibility to comprehensively analyze both targeted and non targeted compounds. The terms used in the literature to differentiate targeted and non targeted analysis are ambiguous and vary between authors. They are classified as

known knowns, known unknowns, unknown unknowns or targeted, non targeted, unknowns [81] and targeted, suspected and non targeted [88]. Stein [89] differentiates the compounds into four categories and called as Rumsfeld quadrants, shown in Figure 3.3. The quadrants are formed on the response to two binary response questions: a) Is the compound expected in the sample b) has the compound been reliably identified. Based on the response the compound can be categorized into one of the four quadrants: known knowns, unknown knowns, known unknowns or unknown unknowns. Known knowns are targeted compounds which are expected and found in the targeted list. Unknown knowns are unexpected but are identified correctly using a comprehensive library. Known unknowns are expected compounds but not found because of the low concentration or not in the library, which represents a failure process. Unknown unknowns are the compound /mass peak unknown to the analyst and also not identified, which is the most challenging. The author proposes to have a library of the spectra for such unknown unknowns which are recurrent and to elucidate the structure for the most commonly appearing compound.

In targeted analysis, the experimental properties (RT, m/z, fragments) of the analytes are characterized with reference standards. The method is normally validated for the

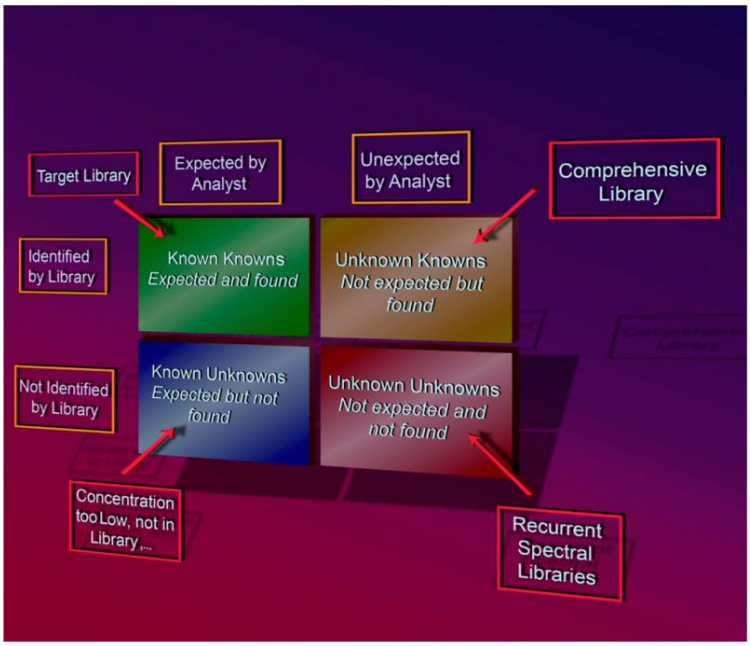


Figure 3.3. Rumsfeld quadrants explaining known knowns, known unknowns, unknown knowns and unknown unknowns in the context of non targeted analysis, reproduced with permission from Stein et.al.[89], Copyright (2012) American Chemical Society.

targeted compounds studying the performance parameters such as recovery, precision and robustness. In a comprehensive analysis, both targeted and non targeted analysis are carried out. A generic non selective extraction is developed and the method is validated for various families of chemical compounds, thus providing an assurance that the method could extract and ionize potentially compounds with different physico chemical properties. The Full MS data can be then treated to search for known unknown/non targeted compounds in a in-house library or a spectraless database [90, 91]. When the chemical peak cannot be assigned to either of a targeted list or non targeted list, then strategies for elucidating the structure will be implemented.

In the field of veterinary drug analysis [92], a comprehensive screening of 110 analytes as targeted compounds with reference compounds, 116 compounds as compounds with exact mass information and different non targeted/semi targeted strategies were developed. Different non targeted strategies include the searching of generic product ions of a class of compounds, relative isotopic abundance and searching for specific neutral losses were applied.

In the field of anabolic substances, identification of an unknown substance was reported based on LC and enzyme-linked immunosorbent assay (ELISA) and LC-QTOF. The sample was found to be positive in ELISA, but negative with QTOF MS for the targeted list of beta agonists. The LC-ELISA fraction which showed positive was then analyzed in full scan mode and the unknown mass with its fragments were identified to be an unknown beta agonist and a structure for the substance was postulated [93]. In another methodology for the analysis of fluroquinolones combining biosensor immunoassay and LC-TOF, three unknown compounds were found [94]. A similar approach for identifying unknown anabolic steroids was also developed adding a accurate mass database [95].

For non targeted screening of pesticides in food, an accurate database for 300 compounds including 400 in source fragment ions generated with TOF has been created [96]. Moreover, method for analysis of pesticides in food, including TOF, QTOF and Orbitrap is being discussed in a review [97]. Group specific fragmentation of six classes of pesticides has been reviewed [98].

In another methodology in pesticide residue analysis, the analytes were categorized into three groups depending on the type of approach followed: targeted when standards are

available and the properties are characterized (retention time, MRM), Non targeted when no compound specific information is available but standards are available elsewhere, unknown analysis when standards are not available. Non targeted compounds can be the impurities or transformation products. The transformation products can be found by the fragmentation degradation relationships. It is suggested that pesticides are fragmented in the same way as they are transformed. [81]. Polgar et. al. [90] developed a pesticide database with accurate masses of precursor ions, 99 metabolites, and 447 diagnostic fragment ions. The analysis was done in a TOF with in source fragmentation. The unknown pesticide metabolites were found by searching for the fragment ions assuming that the metabolites fragment the same way as the parent ones (fragmentation-degradation relationship). The pesticide conjugates were searched with fragment ions and parent pesticide masses. Different examples using the above non targeted screening approaches were illustrated.

Another example is the identification of transformation products of an antibiotic lincomycin in water. Degradation experiments were carried out in a laboratory to characterize the unknown transformation products of lincomycin [99].

Apart from database based screening approaches, metabolic profiling and chemical profiling of Full scan HRMS data are also used as non targeted analysis approaches.

II. Objectives

Some specific objectives in the scope of food safety control as found below were met in this thesis work,

1. Development and validation of an analytical method based on LC-UV for analysis of eight sulfonamides in six types of feeds.
2. Evaluation of different HILIC stationary phases for analysis of aminoglycosides, development and validation of analytical methods for confirmation and quantification of ten aminoglycosides in honey and animal kidney samples
3. Study of a false positive result from LC-MS/MS method for analysing ronidazole in meat and the use of LC-MS/HRMS to avoid suspicion
4. Exploration of different acquisition modes of a hybrid quadrupole Orbitrap mass spectrometer for targeted analysis and development of a method to analyze nine hormones in animal urine
5. Development of a non targeted screening workflow using LC-MS/HRMS data and data analysis tools.

Apart from the method for analyzing sulfonamides and non targeted screening, other methods have been developed in collaboration with Laboratori de Agencia de Salut Publica de Barcelona. The methods were validated according to Decision 2002/657/EC and implemented for routine analysis in accordance with ISO 17025:2005 accreditation.

III. Results and discussion

4. Analysis of sulfonamides in animal feeds with LC - UV

4.1. Introduction

Sulfonamides are a diverse class of synthetic antibiotics having a functional group $–SO_2NH_2$ with chemically related compounds. Sulfonamides are amphoteric in nature characterized by two different pKa values (figure 4.1), which give an indication of the pH values at which the analyte changes its state of ionization. The pKa values can be used to selectively extract sulfonamides from samples using suitable solid phase extraction sorbents. The neutral form is more soluble in organic solvent than the ionic forms. The chemical structure, log K_{ow}, pKa, Chemical Abstracts Service (CAS) and Chemspider register number of the sulfonamides studied are listed in table 4.1. The partition coefficient, log K_{ow} values indicate the hydrophobicity of the analytes. Higher the values of log K_{ow}, higher the organic solvent strength needed to elute the analytes in the reverse phase chromatographic column.

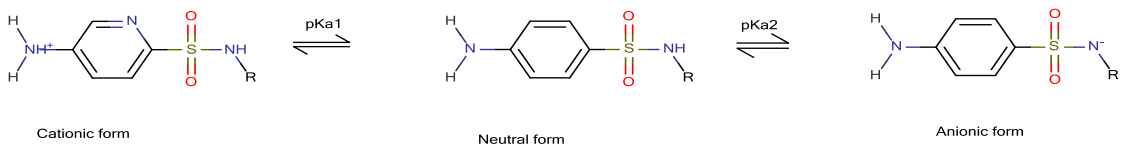


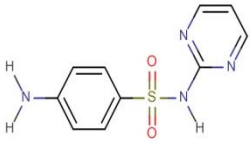
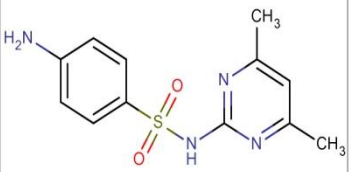
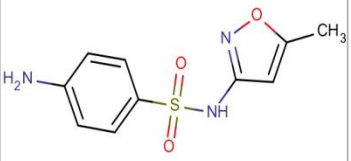
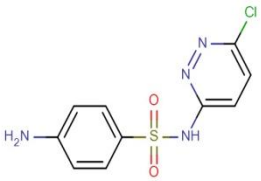
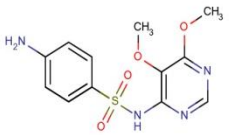
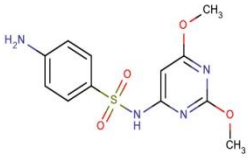
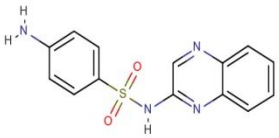
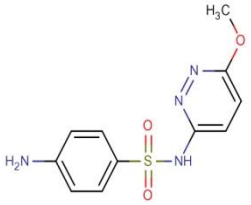
Figure 4.1. General structure and amphoteric nature of sulfonamides

Sulfonamides are bacteriostatic which inhibit growth and multiplication of bacteria and are the oldest antimicrobial group. The first sulfonamide (sulfanilamide) was synthesized in 1936. Nowadays, sulfonamides are used in agriculture, aquaculture, and animal husbandry and rarely in human medicine. In the EU, sulfonamides as antimicrobials are banned to be used for growth promotion in food producing animals since 2006 [100]. However, sulfonamides are authorized to be used in medicated feeds for treatment purposes.

The presence of antimicrobials in feed can be due to authorized use for therapeutic and prophylactic purpose, unauthorized use as growth promoters to increase yield or unintentional cross contamination in the production site where medicated and non medicated feed are prepared in the same production line [101]. Cross contamination is an important reason for the presence of sulfonamides in feed, however recognized as unavoidable even after the application of good manufacturing practices [102]. Controlling

the presence of sulfonamides is important as they pose health hazard such as antimicrobial resistance when they are introduced into the food chain.

Table 4.1. List of chemical structure, molecular formula, log K_{ow}, pKa CAS No. and Chemspider ID of the sulfonamides analyzed.

Analyte	Structure	Molecular Formula	log Kow	pKa	CAS No.	Chemspider id
Sulfadiazine		$C_{10}H_{10}N_4O_2S$	-0,34	pKa1=1,88 ± 0,50 pKa2 = 5,90 ± 0,30	68-35-9	5026
Sulfadimidine		$C_{12}H_{14}N_4O_2S$	0,76	pKa1=1,95 ± 0,50 pKa2 = 7,45 ± 0,50	57-68-1	5136
Sulfamethoxazole		$C_{10}H_{11}N_3O_3S$	0,48	pKa1=1,39 ± 0,10 pKa2 = 5,80 ± 0,50	723-46-6	5138
Sulfachloropyridazine		$C_{10}H_9ClN_4O_2S$	0,31	pKa1=1.88 ± 0,50 pKa2 = 5,90 ± 0,30	80-32-0	6382
Sulfadoxin		$C_{12}H_{14}N_4O_4S$	-0,24	pKa1=1,59 ± 0,37 pKa2 = 6,16 ± 0,50	2447-57-6	16218
Sulfadimethoxin		$C_{12}H_{14}N_4O_4S$	1,17	pKa1=1,30 ± 0,10 pKa2 = 6,21 ± 0,50	122-11-2	5132
Sulfaquinoxaline		$C_{14}H_{12}N_4O_2S$	0,84	pKa1=1,77 ± 0,10 pKa2 = 5,65 ± 0,10	59-40-5	5147
Sulfamethoxypyridazine		$C_{11}H_{12}N_4O_3S$	0,20	pKa1=2,18 ± 0,50 pKa2 = 7,19 ± 0,30	80-35-3	5139

Recently, in 2011[103], the EU has published a scientific report on antimicrobial resistance data for various antimicrobials in zoonotic and indicator bacteria isolates from humans, animals and food. According to the report, sulfonamides along with tetracyclines and ampicillins recorded high resistance levels, while for cephalosporins and fluoroquinolones showed low resistance levels among the model microbes considered in this study.

A recent review by Borras et al, 2011 [101] present the legislative framework in the EU and analytical methodologies for control of antimicrobial agents in animal feed. The authors state that there are very few quality assurance programs, interlaboratory tests and certified reference materials for the analysis of veterinary drugs in feed. This becomes a hindrance for efficient control of antimicrobials in the food chain. JECFA has set an ADI only for sulfadimidine as 0 – 50 μg/kg body weight and there are no limits established for sulfonamides in feed.

Feed is a complex matrix and its composition varies significantly between different types of feeds, which affect the selectivity of the analysis. In the figure 4.1 of the article reported, the composition of various feed can be seen. The composition varies between type of animals and also varies depending on the growth phase of the animal. Borras et.al., [104] evaluated the matrix effects in mass spectrometry by comparing matrix matched standard curves in three different feeds. The comparison of slopes of standard curves showed significant differences depicting that the varying composition affect the calibration curve and thereby the quantitation. The complexity of the various food and feed samples based on matrix ion suppression in mass spectrometry was evaluated by Mol et.al. [62] and order the complexity in the following way, feed > maize > meat > milk > egg > honey.

Most of the methods reported until 2011, for analyzing sulfonamides in feed were developed with liquid chromatography coupled with uv detection (LC-UV), which might be attributed to low economic budgets in feed control laboratories (instrument cost). Moreover, the published methods analyze either one or two feeds. In that sense, a simple LC-UV method with minimal and generic sample preparation to analyze variety of feed matrices deemed to be a necessity and hence developed and reported in the following scientific article.

The objective of this work is to develop a methodology for the determination of eight sulfonamides – sulfadiazine, sulfachloropyridazine, sulfadiazine, sulfadimidine, sulfadoxine, sulfamethoxypyridazine and sulfadimethoxine in six types of animal feed (rabbit, hen, cow, chicken, pig and piglet feed).

4.2. ARTICLE I

Research article

Drug Testing and Analysis

Received: 14 February 2011 Revised: 11 April 2011 Accepted: 12 April 2011 Published online in Wiley Online Library:

(www.drugtestinganalysis.com) DOI 10.1002/dta.296

Development and validation of an LC-UV method for the determination of sulfonamides in animal feeds

P. Kumar and R. Companyó*

A simple LC-UV method was developed for the determination of residues of eight sulfonamides (sulfachloropyridazine, sulfadiazine, sulfadimidine, sulfadoxine, sulfamethoxypyridazine, sulfaquinoxaline, sulfamethoxazole, and sulfadimethoxine) in six types of animal feed. C18, Oasis HLB, Plexa and Plexa PCX stationary phases were assessed for the clean-up step and the latter was chosen as it showed greater efficiency in the clean-up of interferences. Feed samples spiked with sulfonamides at 2 mg/kg were used to assess the trueness (recovery %) and precision of the method. Mean recovery values ranged from 47% to 66%, intra-day precision (RSD %) from 4% to 15% and inter-day precision (RSD %) from 7% to 18% in pig feed. Recoveries and intra-day precisions were also evaluated in rabbit, hen, cow, chicken and piglet feed matrices. Calibration curves with standards prepared in mobile phase and matrix-matched calibration curves were compared and the matrix effects were ascertained. The limits of detection and quantification in the feeds ranged from 74 to 265 μg/kg and from 265 to 868 μg/kg, respectively.

Keywords: sulphonamides; feed analysis; SPE; LC-UV

Introduction

According to a study by the European Federation of Animal Health (FEDESA), in 1999 food-producing animals consumed 4700 tons of antimicrobials in the European Union (EU).[1] Another recent report shows that in 2007, 2478 tons of antimicrobials were sold for use in food and non-food-producing animals in six European countries.[2] The same report states that sulfonamides were the second most commonly used class of antimicrobials for the production of medicated feeds. According to a US Food and Drug Administration (FDA) report, in 2009 food-producing animals consumed 13 067 tons of antimicrobials, of which 517 were sulfonamides.[3]

Aware of the threat of humans developing antimicrobial resistance as a result of the use of antimicrobials in food-producing animals, many countries have established a series of legislations and control measures.[4] In the EU, the use of antimicrobials as feed additives has been prohibited since 2006[5] and Regulation (EC) No. 470/2009 establishes Maximum Residue Limits (MRLs) for antimicrobials in food of animal origin.[6] Also, the Rapid Alert System for Food and Feed (RASFF) has been set up to exchange information about serious risks detected in food and feed. According to RASFF, 122 notifications were issued in 2009 for the presence of veterinary drug residues in a range of food products.[7] Moreover, Commission Decision 2002/657/EC was issued by the EU concerning the performance criteria and procedures for the validation of screening and confirmatory methods.[8]

In the USA, the use of antimicrobials in animals for food production has long been debated between the FDA and livestock producers, and in 2010 this agency released a draft guide on the rational use of these drugs in food-producing animals, as a step to control their application in this context.[3]

Sulfonamides, a diverse class of antimicrobials, are used in animal husbandry for prophylactic and therapeutic purposes and are administered mainly through feed. The concentration of these drugs in medicated feeds ranges between 70 and 800 mg/kg for pig and poultry feeds,[9] which are generally produced in the same production line as unmedicated feeds. The cross contamination of unmedicated feeds during production or transport and the misuse of drugs as feed additives lead to the presence of sulfonamide residues in unmedicated feeds, thus causing the accumulation of these compounds in animals. The MRL for total sulfonamides in food of animal origin is 100 μg/kg.[10] In addition to contributing to the development of antimicrobial resistance, the presence of sulfonamide residues in food-producing animals is of concern because of the potential carcinogenic activity of these drugs in humans.[11]

From an analytical perspective, animal feed is a complex matrix and its composition varies depending on the consumer and to a lesser extent on each production batch. Consequently, each kind of feed has particular characteristics and the varying interfering compounds of the feed make it difficult to develop reliable analytical methods. To overcome this problem, an efficient clean-up step or highly selective detection is required. To the best of our knowledge, there is only one method available to measure multiple sulfonamide compounds along with other compounds in feed matrices; this method is based on liquid chromatography (LC) coupled to tandem mass spectrometry.[12] Other approaches using LC with UV[13–16] or fluorescence detection can also be used to determine either one or two sulfonamide compounds.[14] A few screening methods based on enzyme linked immunosorbent

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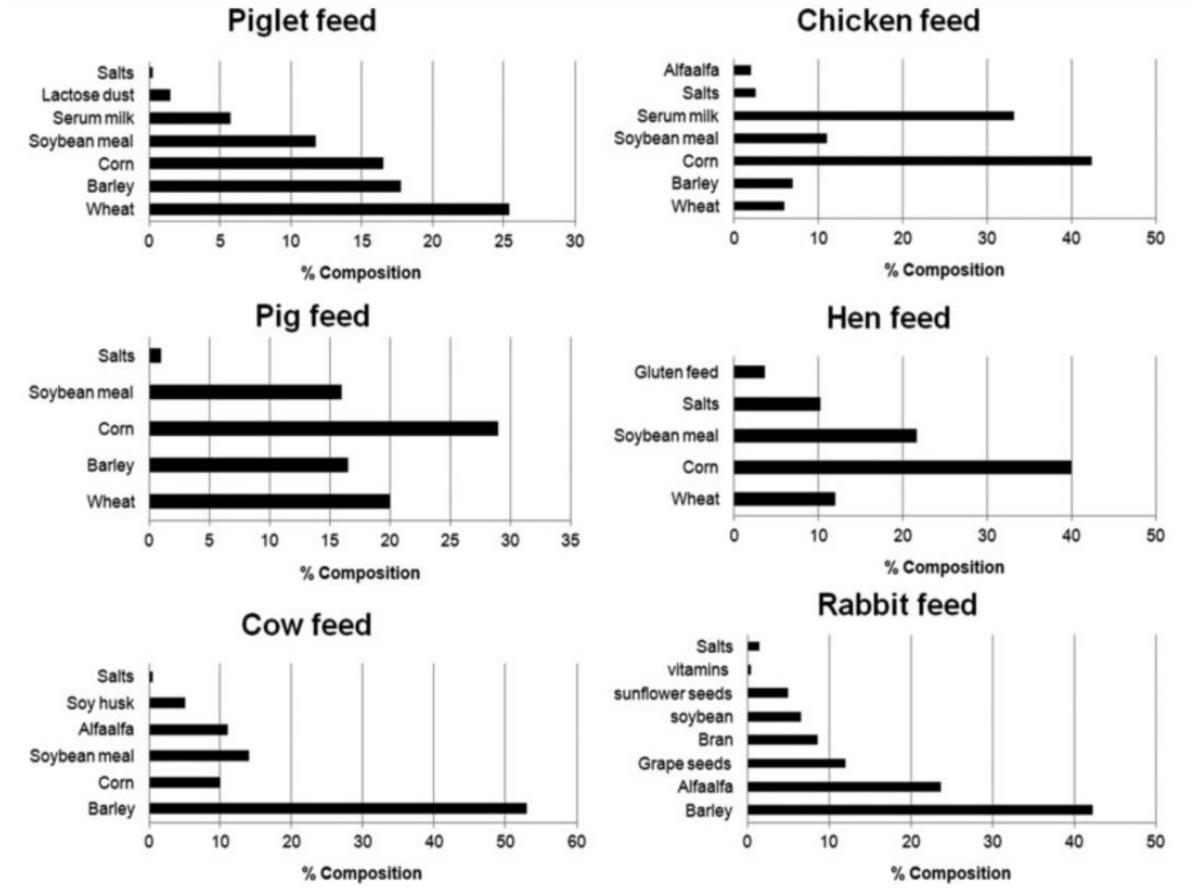


Figure 1. Composition of animal feed samples.

assays (ELISA) have also been proposed.[17–18] Sample treatment usually involves solid-liquid extraction or pressurized liquid extraction (PLE),[12] followed by a clean-up step based on solid phase extraction (SPE).[13,19] In the case of foods of animal origin, many chromatographic methods are available to analyze sulfonamide residues.[11]

Here we studied four distinct SPE phases to achieve efficient clean-up of feed extracts. In addition, we developed and validated an analytical method that applies LC-UV for the determination of sulfachloropyridazine (SCP), sulfadiazine (SDZ), sulfadimidine (SDD), sulfadoxine (SDX), sulfamethoxypyridazine (SMP), sulfaquinoxaline (SQX), sulfamethoxazole (SMX), and sulfadimethoxine (SDM) in various animal feed matrices.

Experimental

Chemicals and solutions

Sulfadiazine, sulfadimidine, sulfamethoxazole, sulfachloropyridazine, sulfadoxin, sulfadimethoxin, sulfaquinoxaline, and sulfamethoxypyridazine, all Vetranal grade, were purchased from Riedel-de Haen (Buchs, Switzerland). Methanol (Merck, Darmstadt, Germany) and acetonitrile (Panreac Quimica SAU, Barcelona, Spain) of LC gradient grade were used. Ultrapure water (Milli Q, Millipore, Molsheim, France) of 18.2 MΩcm^{-1} resistivity was used. All other reagents used were of analytical grade.

A single stock standard solution of all sulfonamides with a concentration of 50 mg/l of each sulfonamide was prepared from solid standards in methanol. The stock solution was stored in amber glass vials at 4 °C for up to 6 months.

Calibration standards (50, 70, 100, 150, and 200 µg/l) were prepared from the stock solution by diluting with initial mobile phase solution (83% buffer: 17% acetonitrile) in a 5-ml volumetric flask.

Matrix-matched calibration standards (50, 70, 100, 150, and 200 µg/l) were prepared by diluting 0.85 ml of blank SPE extracts of the respective feeds and a range of volumes of the stock standard solution (50 mg/l) with the aqueous mobile phase in a 5-ml volumetric flask. Calibration standards were prepared daily.

Preparation of spiked feeds

Pig, rabbit, hen, cow, chicken, and piglet feed samples were kindly provided by the *Associacio Catalana de Fabricants de Pinsos* (ASFAC). They were stored at 4 °C in polyvinylchloride (PVC) flasks. Samples were tested to be free of any analytes, with an LC/MS/MS method. Figure 1 shows the composition of the feeds.

Spiked feed was prepared by mixing blank pig feed with solid sulfonamide standards at a concentration of 2 mg/kg. Initially, a

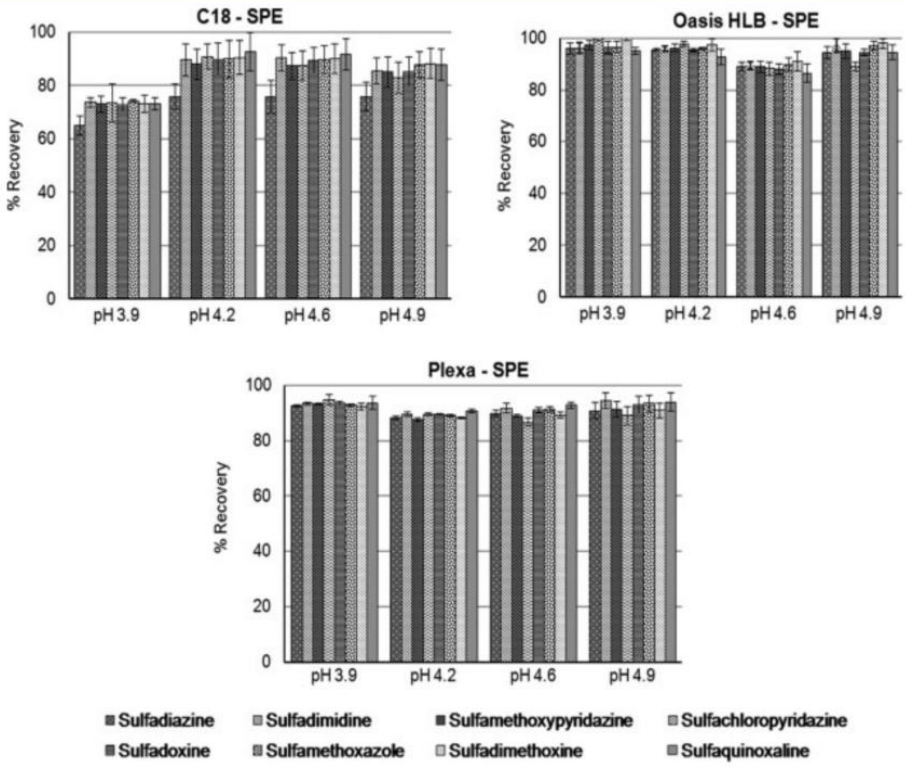


Figure 2. Effect of pH on the retention of sulfonamides in various SPE stationary phases during sample preparation. Standard deviations (n = 3 replicates) are represented as error bars.

sulfonamide concentration of 500 mg/kg material was prepared by adding 62.5 mg of each sulfonamide to 125 g of the pig feed. The mixture was then placed in a PVC flask containing teflon balls and rolled on a rolling table for at least 90 h. Homogeneity of the material was checked by taking samples from several parts of the material and determining the concentration of the sulfonamides by LC-UV. After checking the homogeneity, 10 g of this material was diluted with 90 g of blank pig feed to obtain a new material with a sulfonamide concentration of 50 mg/kg. Homogeneity was checked as described before. The feeds spiked at a concentration of 2 mg/kg were prepared by diluting 4g of the 50 mg/kg pig feed with the respective blank feeds. After homogenization and checking homogeneity, the spiked feed samples were stored in a refrigerator at 4 °C. In these conditions, these materials were stable for up to 18 months.[17]

Extraction and clean-up

To optimize the SPE step of the method, we studied the following four sorbents: Oasis HLB (3 ml × 60 mg cartridges), an N-vinylpyrrolidinedivinylbenzene copolymer sorbent with a hydrophilic-lipophilic balance was purchased from Waters (Milford, MA, USA); Bond Elut C18 (1 ml × 100 mg cartridges), a hydrophobic silica-based sorbent; Bond Elut Plexa (3 ml × 60 mg cartridges), a restricted access material based on a polymeric sorbent with hydroxyl groups on the surface and a hydrophobic polystyrene – divinylbenzene copolymer core; and Bond Elut Plexa PCX (3 ml × 60 mg cartridges), a polymeric sorbent with a strong cation exchange functionality.[20] All Bond Elut cartridges were purchased from Varian (Lakeforest, CA, USA). Rapid Trace® SPE Workstation by Caliper Lifesciences (Hopkinton, MA, USA), an automated SPE system, was used to load sample and solvents onto the cartridge by positive pressure.

Pig feed was used in all the optimization experiments. The effect of pH on sulfonamide retention in C18, Oasis HLB, and Plexa SPE was assessed by loading 500 µg/l standard solutions (in triplicate) prepared at pH 3.9, 4.2, 4.6 and 4.9 and elution with 2 ml of acetonitrile without any washing step. The pH was measured with a Crison GLP21 (Alella, Spain) pH meter equipped with a Crison 52-02 Ag/AgCl combined glass electrode. In Oasis HLB and Plexa, the effect of washing solvent strength on sulfonamide recoveries and clean-up of matrix components was studied by loading blank extracts and standard solutions prepared at pH 3.9. Several solutions of methanol in water (10%, 25%, and 50% *v/v*) were tested as washing solvents.

For Plexa PCX, blank feed extracts and extracts spiked at 500 µg/l, evaporated and reconstituted in 4% phosphoric acid, were loaded (in triplicate) onto the cartridges to assess sulfonamide recovery and clean-up of matrix components. The washing was done in two steps. In the first, 1% formic acid in water was used to keep the sulfonamides in a protonated form while in the second step formic acid in methanol and methanol-water mixtures were studied for their clean-up efficiency. The analytes were eluted with 2 ml of 0.5% ammonia in MeOH: ACN (1 : 1).

The method adopted was as follows: 1 g of spiked or blank feed sample and 10 ml of acetonitrile : water (95 : 5 *v/v*) were added to a 25-ml centrifuge tube, which was then capped, shaken manually for 1 min and then centrifuged at 3500 rpm for 10 min.[17]

A Heraeus Christ Labofuge 400 centrifuge (Osterode am Harz, Germany) with a range of 600 to 6000 rpm was used. An aliquot of 8.5 ml of the supernatant was decanted into a glass test tube, evaporated to dryness at 50 °C for about 30 min in a TurboVap® LV Evaporator Workstation by Caliper Lifesciences (Hopkinton) and reconstituted with 4.25 ml of 4% Phosphoric acid. The sample was then vortexed and placed in the sample rack of the Rapid Trace SPE. The Plexa PCX SPE cartridge was conditioned with 1 ml of methanol, followed by 1 ml of water. Four ml of sample was loaded onto the cartridge at a flow rate of 2 ml/min. The cartridge was washed in two steps with 1 ml of 1% formic acid in water and 1 ml of 2% formic acid in methanol. It was then dried with a nitrogen flow for 0.5 min and eluted with 2 ml of 0.5% ammonia in MeOH: ACN (1 : 1). A portion of 0.85 ml of the eluate was taken and made up to 5 ml in a volumetric flask with pH 4.7 buffer to match initial mobile phase conditions. It was then filtered into injection vials using syringes and 0.45-µm nylon filters from Micron Analitica, S. A. (Madrid, Spain).

Liquid chromatography with UV detection

Chromatographic separation was carried out with an Agilent 1100 (Santa Clara, CA, USA) series liquid chromatography instrument consisting of a degasser, a quaternary pump, an automatic liquid sampler and a diode array detector with tungsten and deuterium lamps. A C8 Inertsil chromatographic column from GL Sciences Inc. (Tokyo, Japan) with a length of 250 mm and diameter of 4.6 mm (5 µm) was used. A pre-column, matching the characteristics of the column and with dimensions of 7.5 × 4.6 mm, was used. Mobile Phase A consisted of 0.01 M aqueous acetic acid – sodium acetate buffer (pH 4.7) and mobile phase B comprised acetonitrile. The LC gradient, at room temperature, began with mobile phase B at 17%, reaching 25% in 15 min, 40% in 20 min and then returning to initial conditions at 25 min with total run time of up to 40 min. The flow rate was maintained at 0.6 ml/min, injection volume was 50 µl and the diode array detector was set at 268 nm.

Method validation

For pig feed, linearity was assessed with matrix-matched standards of 50, 70, 100, 150, and 200 µg/l of sulfonamides, which were prepared and measured on three days. For rabbit, piglet, hen, chicken, and cow feeds, matrix-matched standards at the same levels were prepared and measured on one day. Limits of detection (LODs) and limits of quantification (LOQs) were calculated from ordinary least squares regression data[21] from calibration curves obtained with matrix-matched standards of lower concentrations of 20 to 60 µg/l in pig feed extracts. LOD and LOQ correspond to the analyte concentrations for which the peak area is equal to 3 and 10 times the standard deviation of the intercept, respectively. Inter-day and intra-day precisions of the methodology were assessed by analyzing the pig feed spiked at 2 mg/kg in six replicates on three consecutive days. For rabbit, piglet, hen, chicken, and cow feed samples, intra-day precision was assessed by analyzing six replicates of 2 mg/kg-spiked feeds on the same day. Matrix effect was assessed by comparing the slopes of the calibration curves in solvent (external standards) and matrix-matched calibration curves obtained in the feeds. For this purpose, we used the following *t*-test:[22]

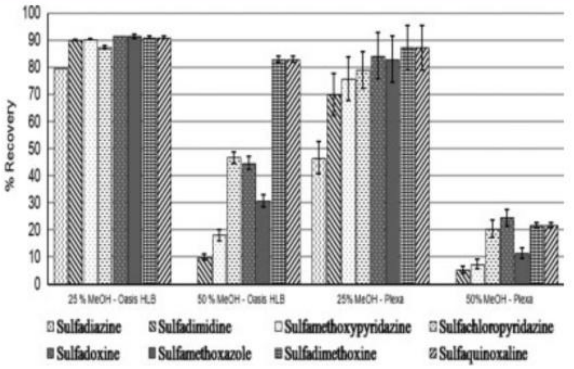


Figure 3. Effect of washing solvent strength on retention of sulfonamides in Oasis HLB – SPE and Plexa – SPE during sample preparation. Standard deviations (n = 3 replicates) are represented as error bars.

$$t_{calc} = \frac{b_1 - b_2}{\sqrt{s_{b_1}^2 + s_{b_2}^2}} \quad (1)$$

where b_1 and b_2 are the slopes and S_{b1} and S_{b2} are the standard errors of slopes of the external calibration curve and matrix-matched calibration curve, respectively. The *t* values calculated were compared with the critical ones at 95% confidence level.

Results and discussion

Extraction and clean-up

Sulfonamides can be extracted from feed matrices by manual shaking for 1 min with acetonitrile containing 5% water.[17] Therefore we adopted this extraction procedure in this study.

The suitability of an SPE phase relies on its capacity to retain the analytes and to remove matrix components to the fullest extent possible. The performance of the three non-functionalized SPE sorbents to retain analytes was studied at several pH values. Under the conditions tested, C18 had less retentive capacity for sulfonamides than Oasis HLB and Plexa, and provided less repeatable results (Figure 2). Recoveries at pH 3.9 in Oasis HLB and Plexa were slightly higher than at the other pH values. Indeed, the average recoveries for all the analytes at pH 3.9 were 98% (Oasis HLB) and 94% (Plexa) compared with 95–98% (Oasis HLB) and 89–92% (Plexa) at the other pH values. Thus pH 3.9 was selected for further studies.

In order to ascertain the clean-up capacity of the sorbents, several washing solutions were assessed. A preliminary study using a 10% solution of methanol in water showed that SDZ was washed off in C18 cartridges. Therefore C18 was not studied further. When a 25% aqueous solution of methanol was applied as washing solvent, Oasis HLB gave similar recoveries for all analytes and more repeatable results than Plexa (Figure 3). When 50% methanol was used, significantly lower recoveries were obtained for most analytes in both cartridges. Moreover, chromatograms of blank feed extracts showed that the removal of interferences (matrix peaks) improved in Oasis HLB (Figure 4A), with an increase in the methanol percentage; however, no improvement was detected in Plexa (Figure 4B). On the basis of these observations, we concluded that Oasis HLB shows the highest clean-up capacity of the three non-functionalized phases.

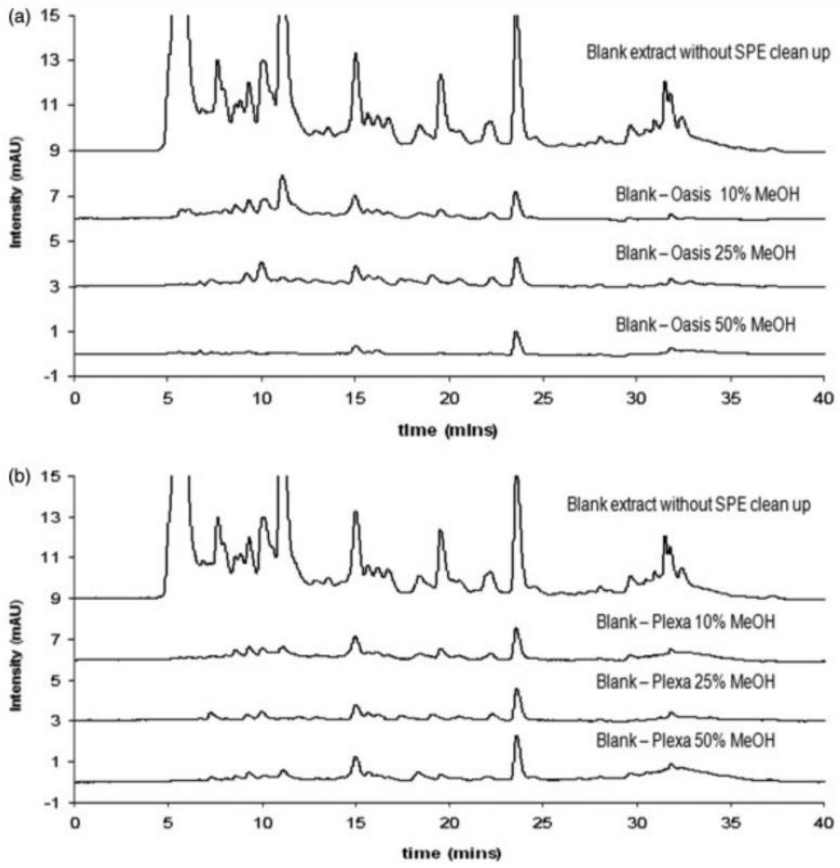


Figure 4. (A) Chromatograms of blank pig feed samples after Oasis HLB – SPE and (B) Plexa – SPE during sample preparation, measured at 268 nm wavelength.

Because sulfonamides become protonated at pH values below 4, we also studied a clean-up approach based on an SPE cation exchange sorbent, Plexa PCX. In this case, after solvent evaporation, the extracts were reconstituted with a 4% phosphoric acid solution and loaded onto the cartridges. A first washing step with 1% formic acid did not cause any loss of analytes. In order to improve extract clean-up, a second washing step with acidic solutions containing methanol was applied. In contrast to the other phases, an increase in the methanol percentage did not lead to greater loss of analytes (Figure 5). Moreover, 2% formic acid in MeOH produced cleaner extracts than 2% formic acid in MeOH: water mixtures (Figure 6).

Finally, pig feed spiked at 2 mg/kg was analyzed in six replicates using the optimized Oasis HLB and Plexa PCX SPE approaches. Plexa PCX cartridges gave recoveries ranging between 47% and 66% with RSD% between 4% and 15% while those for Oasis HLB cartridges ranged between 47% and 77% with RSD% between 5% and 21% (Figure 7). Although Plexa PCX produced lower recovery values than Oasis HLB, it gave more repeatable results for all the analytes and thus, Plexa PCX was finally selected.

Method validation

Calibration curves with matrix-matched standards of the six feeds were prepared in the range 50–200 µg/l. Table 1 shows the linearity parameters of the calibration curves obtained in pig feed. Curves corresponding to the other feeds showed similar linearity parameters. Figure 8A shows the chromatogram of a 200 µg/l matrix-matched standard in pig feed.

LOD and to a lesser extent LOQ are not robust parameters. Depending on the method applied to estimate LOD, mathematical artifacts without analytical significance are sometimes obtained. These artifacts are attributed to the fact that LOD and LOQ must be calculated from calibration curves at the lowest concentration levels of the method linear range, where the precision of signal values is low. Here we applied the approach based on signal-to-noise ratio and the one based on standard deviation of the intercept of the calibration curve. The first approach gave LOD values between two and five times lower than the second. On the basis of our practical experience using this method, we concluded that the values obtained from the regression data were more realistic. They were therefore adopted and consigned in Table 1. Figure 8B shows a chromatogram of a 20 µg/l matrix-matched standard overlaid on a chromatogram of a blank feed extract. This concentration was close to the LOD for most of the analytes, except SDZ and SMX, which showed twice the LOD values. The analyte peaks were well distinguished from the matrix peaks and their areas were measured for quantification purposes.

Precision and trueness expressed as recovery values are summarized in Table 2. Matrix matched calibration standards

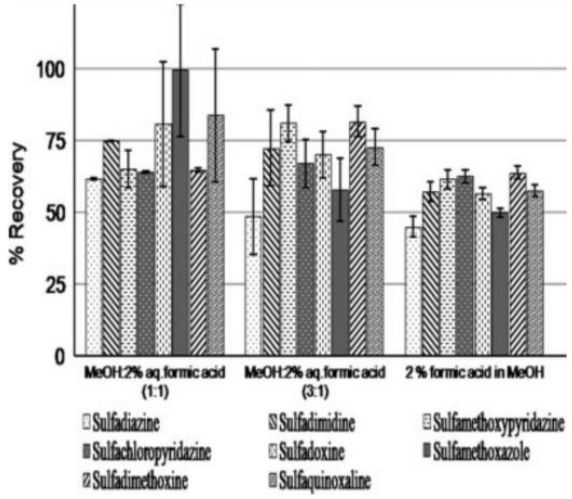


Figure 5. Effect of washing solvent in second washing step on recovery % using Plexa PCX – SPE. Standard deviations (n = 3 replicates) are represented as error bars.

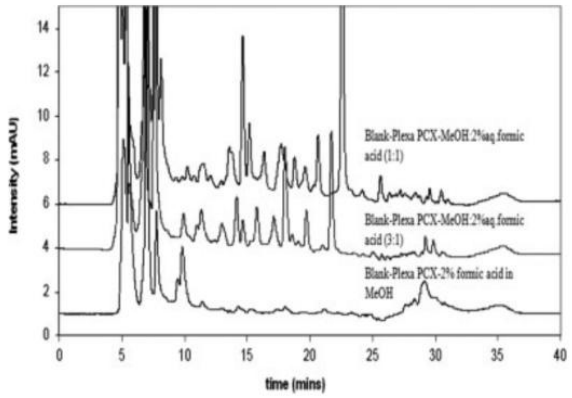
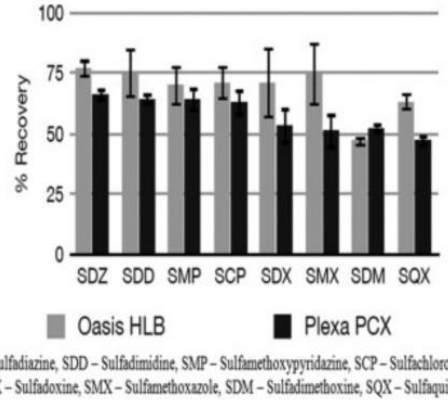


Figure 6. Chromatograms of blank pig feed samples after Plexa PCX-SPE with a range of washing solvents in a second washing step, measured at 268 nm wavelength.

were used for quantification. Table 2 shows that the average of the recovery values of all sulfonamides in each feed varied considerably between matrices. This finding could be attributed to the difference in the composition of the feed.

Analysis of complex samples such as animal feeds calls for the consideration of possible effects of sample matrix on the analytical signal. Matrix-matched calibration standards are generally used for quantification purposes in order to compensate for the effects of matrix mismatch between samples and standards. The use of calibration standards for solvents simplifies the analytical methodology and they can be used for quantification provided there are no significant matrix effects.[23]

In order to ascertain the presence of matrix effects, we compared the slopes of the calibration curves in solvent with those obtained with matrix-matched standards. No significant differences at 95% confidence level between the slopes of any analyte were observed in pig and rabbit feed (Table 3). In the case of chicken feed, only SQX showed a significant difference. Matrix effects were



SDZ – Sulfadiazine, SDD – Sulfadimidine, SMP – Sulfamethoxypyridazine, SCP – Sulfachloropyridazine, SDX – Sulfadoxine, SMX – Sulfamethoxazole, SDM – Sulfadimethoxine, SQX – Sulfaquinoxaline.

Figure 7. Sulfonamide recoveries and standard deviation of the Oasis HLB and Plexa PCX SPE approaches assessed with spiked pig feed (2 mg/kg). Standard deviations (n = 3) are represented as error bars.

Table 1. Linearity parameters (n = 3 days, 5 levels), LODs and LOQs of the sulfonamides in LC-UV calculated from ordinary least square regression data using matrix-matched standards in pig feed

	Slope	Intercept	R^2	LOD in extract µg/l	LOQ in extract µg/l	LOD in sample µg/kg	LOQ in sample µg/kg
SDZ	0.3956	−2.4985	0.9998	13	45	191	662
SDD	0.3544	−3.2839	0.9985	5	18	74	265
SMP	0.3272	1.0656	0.9964	10	33	147	485
SCP	0.2563	−1.7394	0.9999	9	29	132	426
SDX	0.3687	−4.1828	0.9966	5	18	74	265
SMX	0.465	−6.661	0.9996	18	59	265	868
SDM	0.3284	−2.3985	0.9983	5	18	74	265
SQX	0.4047	−2.3437	0.9997	5	18	74	265

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more severe in the other three feeds studied. When matrix effects cause non-acceptable errors in analyte quantification, the method of standard additions should be used for quantification when blank feeds are not available to prepare matrix-matched standards. To assess the effect of matrix mismatch in quantification, feeds spiked at a concentration of 2 mg/kg (six replicates) were analyzed and quantified with both matrix-matched calibration curve and external calibration curve. Maximum relative errors in the concentrations of analytes quantified with external standards were 32% in hen and piglet feed and 46% in cow feed.

Conclusions

Of the few methods reported for the determination of sulfonamides in animal feeds, all of them address the analysis of only one[12–13,17–18] or two[15–16] kinds of feeds, mostly those used for pigs. Moreover, except for a recent study[12] devoted to LC-MS/MS analysis of 22 veterinary drugs belonging to the β-lactam and sulfonamide families, the remaining ones deal with the analysis of one or two compounds. With regard to sample treatment,

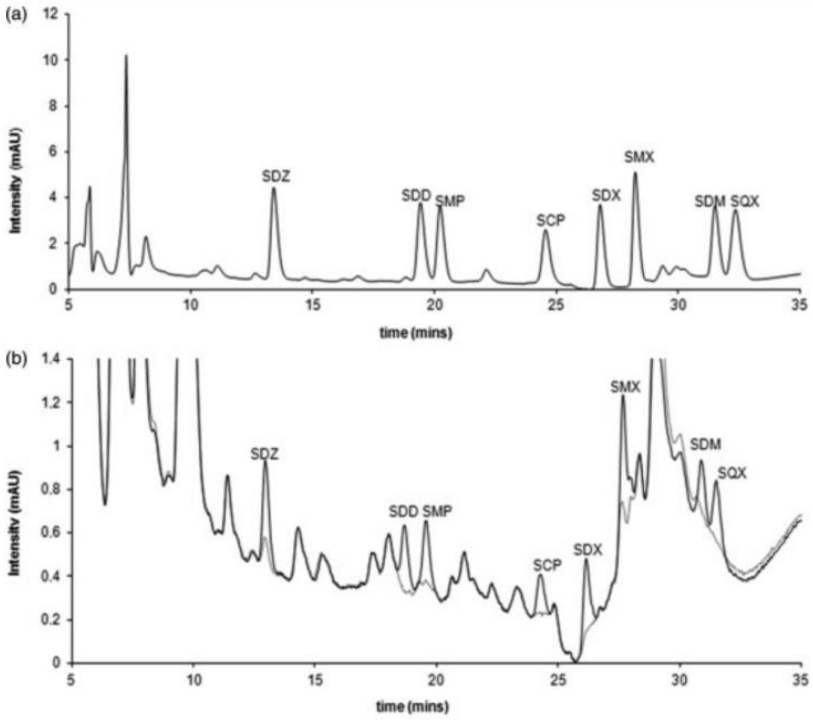

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Figure 8. (A) Chromatogram of a 200 µg/l matrix-matched standard in pig feed and (B) chromatogram of a 20 µg/l matrix-matched standard overlaid on blank pig feed sample, measured at 268 nm wavelength.

Table 2. Intra-day precision (n = 6), inter-day precision (n = 18) and accuracy of the whole LC-UV method assessed with the spiked feed (2 mg/kg) in several feed matrices prepared by homogenising blank feeds with solid sulfonamide standards

	Pig feed		Rabbit feed	Piglet feed	Hen Feed	Chicken feed	Cow feed
	Recovery % (Intra-day precision - RSD%)	Inter-day Precision (RSD%)	Recovery % (Intra-day precision - RSD%)	Recovery % (Intra-day precision - RSD%)	Recovery % (Intra-day precision - RSD%)	Recovery % (Intra-day precision - RSD%)	Recovery % (Intra-day precision - RSD%)
SDZ	66(4)	7	60(5)	52(3)	61(4)	56(5)	52(6)
SDD	64(4)	11	51(14)	37(4)	67(5)	56(12)	48(11)
SMP	64(8)	18	58(6)	47(12)	64(9)	66(10)	51(9)
SCP	63(9)	17	59(11)	68(8)	55(7)	67(4)	55(14)
SDX	53(14)	16	75(20)	42(11)	63(17)	52(19)	46(20)
SMX	51(15)	14	43(19)	40(17)	45(12)	45(15)	40(10)
SDM	52(4)	14	48(5)	38(3)	52(5)	46(3)	43(5)
SQX	47(6)	11	38(8)	33(3)	42(4)	38(7)	43(11)
Average recovery %	58		54	45	56	53	47

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Croubels *et al.*[13] applied cation exchange SPE to purify sulfadiazine extracts, achieving a recovery of 31%. After performing a pressurised liquid extraction, Kantiani *et al.*[12] used Oasis HLB cartridges but only relative recoveries (corrected by the surrogates) were reported. Here we propose a method that comprises a rapid solid-liquid extraction and an SPE clean-up with Plexa PCX cartridges. This approach is suitable for the determination of eight sulfonamide residues in six feed matrices. It can also be used for quality control and inspection purposes for feed containing higher concentrations of these drugs, such as in medicated feeds. Concerning the calibration, external standards can be used in the analysis of pig and rabbit feed, as studied in the present work.

Table 3. Study of matrix effect by comparing slopes of external calibration curve (prepared in solvent) and matrix- matched calibration curve (prepared in blank SPE eluates). (+) Presence of matrix effect (slopes of two regression lines are significantly different at 95% confidence level). (−) No matrix effect (slopes of two regression lines are not significantly different at 95% confidence level). Measurement was done with LC-UV

	Pig feed	Rabbit feed	Piglet feed	Hen feed	Chicken feed	Cow feed
SDZ	−	−	+	+	−	+
SDD	−	−	−	−	−	+
SMP	−	−	+	−	−	+
SCP	−	−	+	+	−	+
SDX	−	−	+	−	−	−
SMX	−	−	+	−	−	+
SDM	−	−	−	−	−	+
SQX	−	−	+	+	+	+

SDZ – Sulfadiazine, SDD – Sulfadimidine, SMP – Sulfamethoxypyridazine, SCP – Sulfachloropyridazine,
SDX – Sulfadoxine, SMX – Sulfamethoxazole, SDM – Sulfadimethoxine, SQX – Sulfaquinoxaline.

In contrast, matrix-matched standards or the standard additions approach should be used with piglet and cow feeds. For hen and chicken feeds, only a few analytes would require these approaches. Given its simplicity and the instrumentation involved, the proposed method can be implemented in most routine laboratories as a quantitative method, provided that confirmation of analyte identity is not required. According to EU legislation,[8] a confirmatory analysis calls for the use of LC-MS/MS, which is a more powerful but also more expensive technique.

Acknowledgements

This study was supported by the Spanish Ministerio de Ciencia e Innovación (Project AGL 2008-05578-C05-03). The authors acknowledge the Associacio Catalana de Fabricants de Pinsos (ASFAC) for providing feed samples. P Kumar thanks the Generalitat de Catalunya for an FI grant (Grant Number 2010 FI-B 00676).

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4.3. Discussion

Extracting solution should provide high recovery of analytes without coextracting the interfering compounds. Two extracting solutions, acetonitrile (5% water) and 0.05 M hydrochloric acid HCl (10 ml) were tested. 1 g of blank feed sample was extracted with the two extracting solutions. The chromatograms of the extracts in figure 4.2 show that acetonitrile extracts are cleaner than HCl extracts and hence acetonitrile (5% water) was chosen as extracting solution.

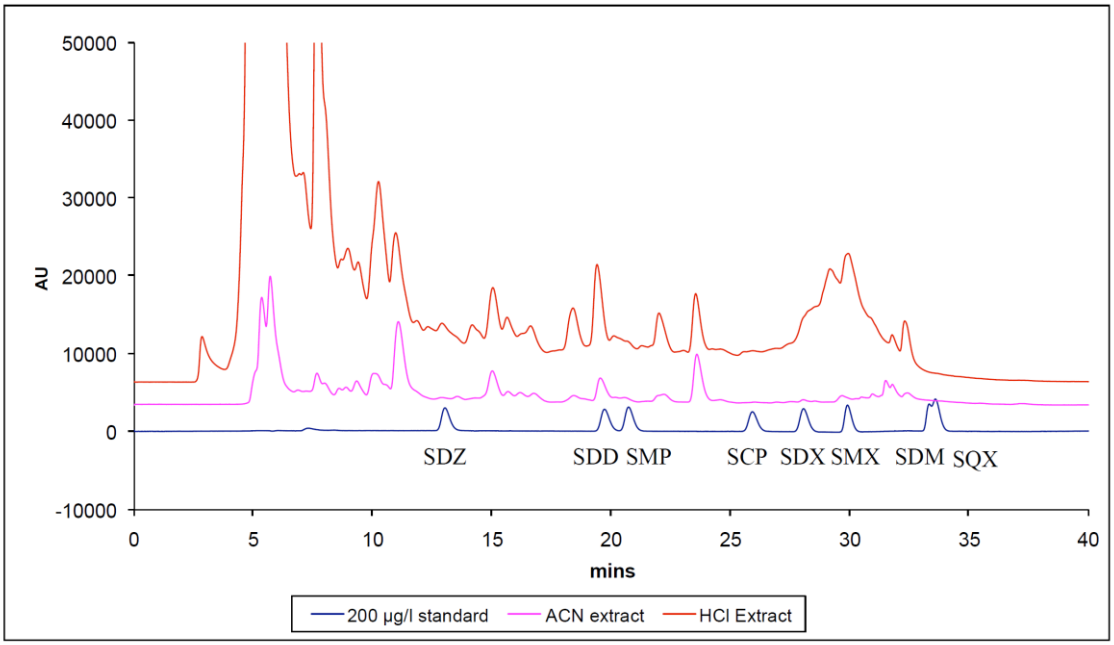


Figure 4.2. Chromatograms of blank feed sample extracted with 0.05 HCl and blank feed extracted with ACN (5% water)

For the cleanup step, solid phase extraction using three non functionalized sorbents and one functionalized sorbent were studied. Non functionalized sorbents are C18, Oasis HLB and Bond Elut Plexa and Plexa polymeric cation exchange (PCX) as mixed mode strong cation exchange sorbent is the functionalized sorbent. The results of the optimization study are summarized in table 4.2. Feed being a complex matrix, in the clean up step analytes have to selectively extracted washing out as much as interferences possible. In Plexa PCX, it was observed that washing solution containing only methanol removed the interferences effectively without losing considerably the analytes. In case of other sorbents, when proportion of methanol was increased in the washing solution, the recovery of analytes reduce significantly.

Table 4.2. Summary of the results of studying various SPE sorbents.

Sorbent	Description	Results
C18	silica based sorbent with hydrophobic properties	Less retentive capacity for sulfonamides
Oasis HLB	polymeric sorbent with hydrophilic lipophilic balance (HLB)	Analyte loss during washing with high percentage of methanol
Bond Elut Plexa	Restricted access packing with size exclusion and reversed phase mechanisms	Analyte loss during washing with high percentage of methanol
Bond Elut Plexa PCX	Mixed mode ion exchanger with strong cation exchange sites and hydrophobic properties	No analyte loss during washing with high percentage of methanol

A general scheme of the analytical methodology and the parameters optimized in this work, are shown in figure 4.3. Spiked extracts were used to evaluate the clean up and recovery of extraction considering the uncertainties of SPE and LC steps. A spiked feed of 2 mg/kg concentration prepared in the laboratory was used for analyzing the recovery of the whole method considering the uncertainty of all the steps in the methodology. As shown in the figure 4.3, in this work, extracting solvents, possibility of avoiding the evaporation step and the solid phase extraction step were optimized.

During method validation LOD and limit of quantitation (LOQ) was estimated using two methods. One is using noise data where LOD and LOQ correspond to 3 and 10 times the standard deviation of the concentration of noise. Another method was based on standard deviation of the intercept of the calibration curve, and LOD and LOQ correspond to 3 and 10 times the standard deviation of the intercept of calibration curve. The LOD and LOQ values calculated from both the methods are shown in table 4.3. The values calculated based on noise data is lower than regression data.

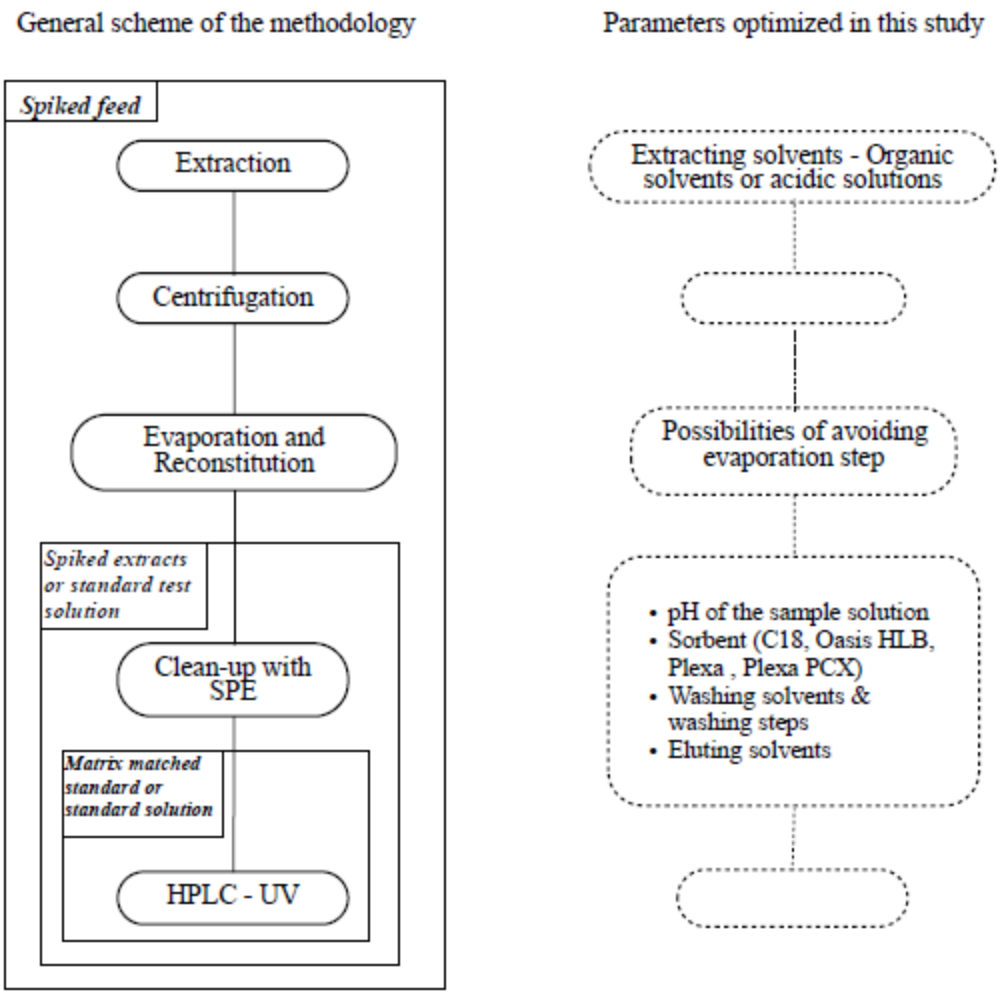


Figure 4.3. Illustrating the use of spiked feed, spiked extracts and standard solutions for optimization of the method.

The influence of matrix interferences on UV detection of analytes is mainly the selectivity. More the interferences at the elution time of analytes, more the chances that interferences would be detected as analyte and thereby less the selectivity of the method. This would affect the calibration and thus the quantitation. Matrix effects were assessed by comparing slopes of external standard calibration curve and matrix matched calibration curve of all the analytes in various feeds. According to the influence of matrix interferences in analyte calibration curve, the feeds can be ordered as follows pig and rabbit feed < chicken feed < hen feed < piglet feed < cow feed.

Table 4.3. LOD and LOQ values estimated by two different methods.

Analyte	*LOD (µg/l)	*LOQ (µg/l)	**LOD (µg/l)	**LOQ (µg/l)
Sulfadiazine	4.9	16.3	13	45
Sulfadimidine	3.2	10.7	5	18
Sulfamethoxypyridazine	1.8	6	10	33
Sulfachloropyridazine	1.9	6.4	9	29
Sulfadoxine	12.2	40.6	5	18
Sulfamethoxazole	9.4	31.2	18	59
Sulfadimethoxine	3.6	12	5	18
Sulfaquinoxaline	4.2	14	5	18

* calculated based on standard deviation of noise data
** calculated based on standard deviation of intercept of calibration curve

Table 4.4. Summary of performance characteristics of this method.

LOD in sample	74-265 µg/kg
LOQ in sample	265-868 µg/kg
Extraction recoveries	47-66%
Intraday Precision	4 – 15%
Interday Precision	7-18%

The performance characteristics of this method are summarized in table 4.4. Apart from the methods from literature reviewed in the scientific article, a method based on LC – UV to analyze ten sulfonamides in feeds has been reported by Iammarino et. al., in 2011 [105]. LOQs of the method for sulfonamides in feed range between 1290 and 2130 µg/kg which are higher than the LOQs of the current method (table 4.4). Moreover, in this current method, an evaporation step is avoided after SPE elution step which can provide high throughput while analyzing routine samples.

In order to increase the selectivity, methods based on mass spectrometric detection especially, high resolution mass spectrometry would be preferable due to the possibility to measure accurate masses and thereby discriminate the matrix interferences. However,

matrix effects in the ion source have to be addressed. Mass spectrometry based methods for analyzing sulfonamides in feed are scarce in comparison to methods testing food. Table 4.5 summarizes the methods based on liquid chromatography coupled with low resolution mass analyzers - triple quadrupole (QqQ), quadrupole linear ion trap (QIT) and high resolution mass analyzers - quadrupole TOF (QqTOF), Orbitrap. It could be noted that LOQ in sample of the MS based methods are much lower than the LC-UV method. The methods based on low resolution QqQ mass analyzers are published more than methods based on high resolution mass analysers. This could be because, nowadays QqQ mass analyzers are available in many control laboratories and the use of high resolution mass analyzers for regulatory purpose is not common yet. The trend in developing MS based methods is to analyze multi class, multi family compounds in the same method analyzing more than 100 analytes using a generic extraction with acidified organic/aqueous solution. For example, Aguilera-Luiz et.al. [106] analyzed 364 analytes in the same method applied to three different types of feed. In that way, high throughput of analysis can be achieved and when coupled to high resolution mass spectrometers, unambiguous results can be attained. However, confirmation criteria to use HRMS based methods are often debated and adequate criteria like mass accuracy has not been specified by Decision 2002/657/EC. The method based on LC-Orbitrap [107] use precursor ion of analytes for identification and quantification with mass accuracy tolerance to be < 5 ppm and the fragment ions were not monitored. Apart from targeted analysis, HRMS based methods have possibility to carry out non targeted analysis as Full scans can be measured. Non targeted analysis could be significant in case of feed analysis where industrial contaminants like dioxins, mycotoxins and pesticide residues are often reported in feed [108].

Table 4.5. Summary of liquid chromatography mass spectrometry based methods that analyze sulfonamides in animal feed

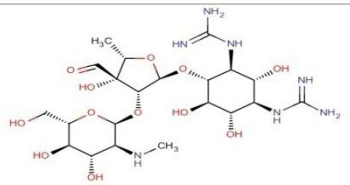
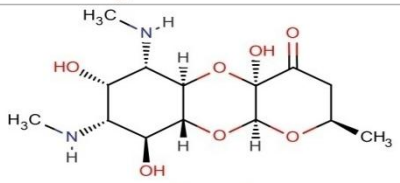
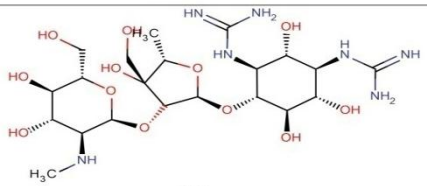
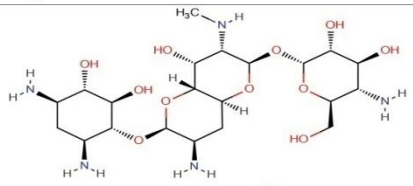
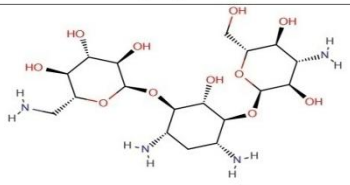
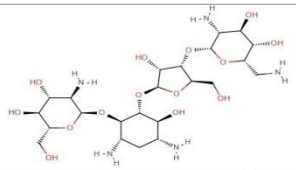
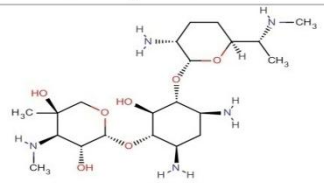
Sl.No.	Analytes	Sample	Extraction	Chromatography and detection	LOQ	Working range	Interday Precision (RSD%)	Reference and Year
1	15 sulfonamides along with 157 pesticides, mycotoxins, plant toxins, and veterinary drugs	Horse feed and other food samples	Extraction with water and acidified organic solvent	100 mm × 2.1 mm i.d., 1.7 µm BEH-C18 column coupled with QqQ	10 µg/kg	10 - 250 µg/kg	11-28 %	Mol et. al., 2008[62]
2	Sulfamethazine, sulfadiazine, along with 12 other veterinary drugs	Pig and poultry feed	Extraction with acetonitrile	100 × 2 mm, 3 µm particle size, Luna C18 column coupled with QqQ	NA	100 - 1000 µg/kg	20 and 28%	Cronly et.al., 2010[109]
3	7 sulfonamides, along with 11 penicillins and cephalosporins	Bovine feed samples	Pressurized Liquid extraction and solid phase extraction	50 mm x 2 mm, 4 µm, C12 Phenomenex Hydro-RP coupled with Q linear ion trap	0.31-2.92 µg/kg	25 - 300 µg/kg	3 - 11%	Kantiani et.al., 2010[110]
4	7 sulfonamides, along with 11 penicillins and cephalosporins	Bovine feed samples	Pressurized Liquid extraction and online solid phase extraction	50 mm x 2 mm, 4 µm, C12 Phenomenex Hydro-RP coupled with Q linear ion trap	0.26 – 5.79 µg/kg	10-500 µg/kg	2-8%	Kantiani et.al., 2010[111]
5	17 sulfonamides, along with 347 pesticides and veterinary drugs	Chicken. Hen, rabbit and horse feed samples	Extraction with acidified acetonitrile	100 mm × 2.1 mm i.d., 1.7 µm BEH-C18 column coupled with QqTOF	2.5 – 12.5 µg/kg	1-150 µg/kg	6 - 25%	Aguilera-Luiz et.al., 2013[106]
6	11 sulfonamides and 39 other veterinary drugs	Pig, poultry and cattle feeds	Extraction with acidified organic solvent	100 mm × 2.1 mm; 1.7 µm, Kinetex XB-C18 column coupled with QqQ	7.7 – 66.7 µg/kg	25-1000 µg/kg	10 - 20 %	Borras et.al., 2013[104]
7	10 sulfonamides and 38 other veterinary drugs	Pig, poultry and cattle feeds	Extraction with water and acidified organic solvent	50 mm × 3.2 mm, 5 µm Altima HP C18 analytical column, coupled with Orbitrap	NA	10-500 µg/kg	NA	Kaklamanos et.al., 2013[107]

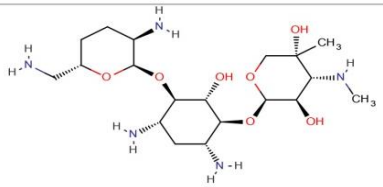
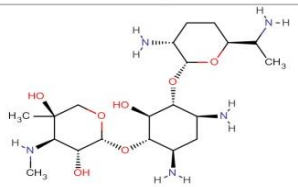
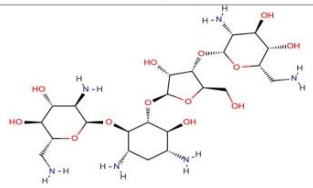
5. Analysis of aminoglycosides with HILIC – MS/MS in honey and animal kidney

5.1. Introduction

Aminoglycosides are a class of antimicrobials administered to food producing animals for treatment purposes. Their presence in food producing animals has to be controlled because of health hazards like ototoxicity and nephrotoxicity. In addition, as antibiotics, their overuse can cause antimicrobial resistance.

Table 5.1. List of the aminoglycosides studied, their chemical structure, molecular formula and log K_{ow}.

Analyte	Structure	Molecular Formula	log Kow
Streptomycin		$C_{21}H_{39}N_7O_{12}$	-1,75
Spectinomycin		$C_{14}H_{24}N_2O_7$	-0,82
Dihydrostreptomycin		$C_{21}H_{41}N_7O_{12}$	-3,38
Apramycin		$C_{21}H_{41}N_5O_{11}$	-3,43
Kanamycin A		$C_{18}H_{36}N_4O_{11}$	-6,70
Paromomycin		C23H45N5O14	-0,58
Gentamycin C1		$C_{21}H_{43}N_5O_7$	-1,48

Gentamycin C1a		$C_{19}H_{39}N_5O_7$	**-2,37**
Gentamycin C2/C2a		$C_{20}H_{41}N_5O_7$	**-1,95**
Neomycin B		$C_{23}H_{46}N_6O_{13}$	**-5,96**

Aminoglycosides are made up of glycosides attached with amino groups (-NH2). They have multiple ionization sites and are highly polar. The chemical structures, molecular formulae and the partition coefficient ($logK_{ow}$) of the studied aminoglycosides are listed in table 5.1. It can be noted that the values of partition coefficients are in negative showing that they are highly polar.

The ADI values set by JECFA and CVMP and their MRLs in animal kidney are summarized in table 5.2. Although honey is a matrix where many prohibited antimicrobials are reported to be found, there has not been any MRL set by Codex or the EU for antimicrobials in this matrix. Community Reference Laboratory (CRL) at Fougeres set an MRPL only for streptomycin in honey as 40 µg/kg [112, 113].

Aminoglycosides being polar compounds, the reverse phase chromatography would require use of strong ion pairing agents like heptaflourobutyric acid (HFBA) to achieve retention. The aminoglycosides are paired in solution with the ion pairing agent and the neutral complex undergoes partitioning with the reversed phase stationary phase in order to achieve chromatographic retention (figure 5.1). But this approach is generally not preferred due to strong ion suppression in MS caused by ion pairing agent and also it require extensive cleaning to remove them from the instrument [114].

Table 5.2. ADI, Codex MRL and EU MRL for aminoglycosides in kidney

Analyte	ADI µg/kg body wt JECFA	Codex MRL in kidney µg/kg	ADI µg/kg body wt CVMP	EU MRL in kidney µg/kg
Streptomycin	0-50*	1000*	25	1000
Dihydrostreptomycin				1000
Spectinomycin	0-40	5000		5000
Apramycin			250	20000
Paromomycin			34	1500
Kanamycin A				2500
Gentamycin C1				
Gentamycin C2/C2a	0-20**	5000**	100**	750**
Gentamycin C1a				
Neomycin B			60	5000

*** Streptomycin and dihyrostreptomycin combined. ** - Genta C1, C2/C2a and C1a combined**

An alternative to ion pairing - reverse phase chromatography of aminoglycosides is to attain retention using HILIC. HILIC is a variant of normal phase chromatography, where highly polar compounds can be retained. The stationary phases can be grouped into neutral, polar or ionic phases depending on the structural variations of the functional groups. In the last decade, HILIC has progressed and has been utilized in many applications and the types of commercially available HILIC columns are ever growing.

Unmodified bare silica gel is a typical HILIC stationary phase, where type A, B and C silica gels are used [115]. Type A silica is prepared by precipitation from silicates and contain metal impurities. Type B silica is prepared by aggregation of silica in air, containing low amount of metals. Type C silica contain a hydrosilated surface with non polar Si-H groups and less than 5% of silanols thus having less retention than other silica types. Other commonly used HILIC stationary phases are amino, amide, diol, zwitterionic (sulfoalkylbetaine), polyscuccinnamide and cyanopropyl which are depicted in figure 5.2.

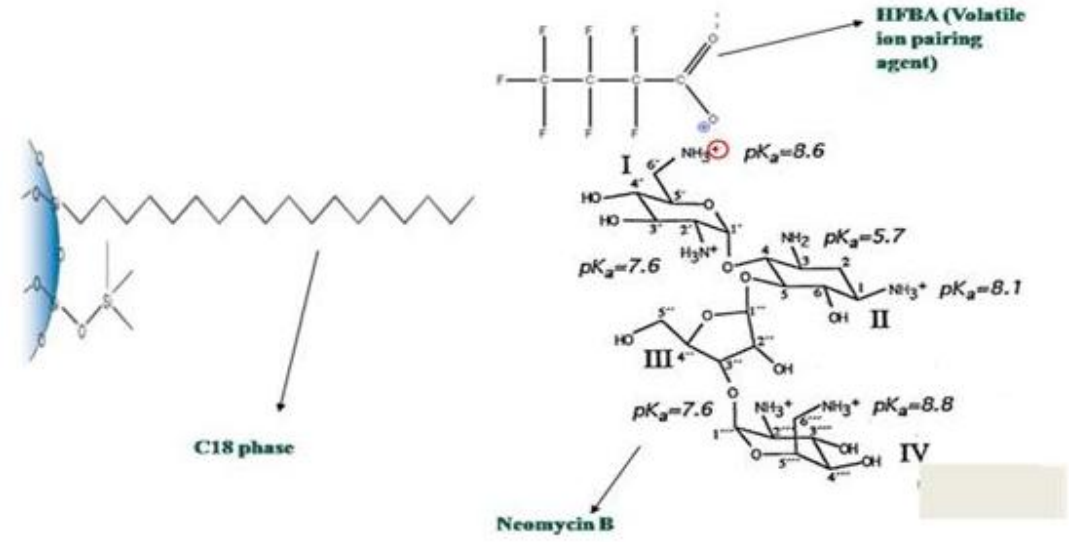


Figure 5.1.Retention mechanism in ion pairing reverse phase chromatography.

A typical mobile phase for HILIC contains water and a miscible polar organic solvent. The solvent strength in HILIC can be summarized as follows : acetone < isopropanol < acetonitrile < ethanol < dioxane < methanol < water. HILIC separations are carried out in isocratic mode or with gradients starting with a high percentage of an organic solution and ending with a high percentage of an aqueous solution. This is advantageous while coupling to MS, where high organic content in the mobile phase leads to rapid evaporation of solvents in electrospray ionization and thereby increases the sensitivity of the analytes. It is commonly believed that the mobile phase forms a water-rich layer on the surface of the polar stationary phase. Ionic additives like ammonium acetate and ammonium formate are used to control pH and ionic strength, thereby affecting the retention. pH must be adjusted to ensure that the ionisable analytes will be in a single form.

Retention and separation mechanisms in HILIC depend on the functional group of stationary phase, mobile phase characteristics (water content, pH, ionic strength) and properties of the analytes. The analyte retention mechanism is multimodal involving: partitioning between the water layer formed in the stationary phase and the organic

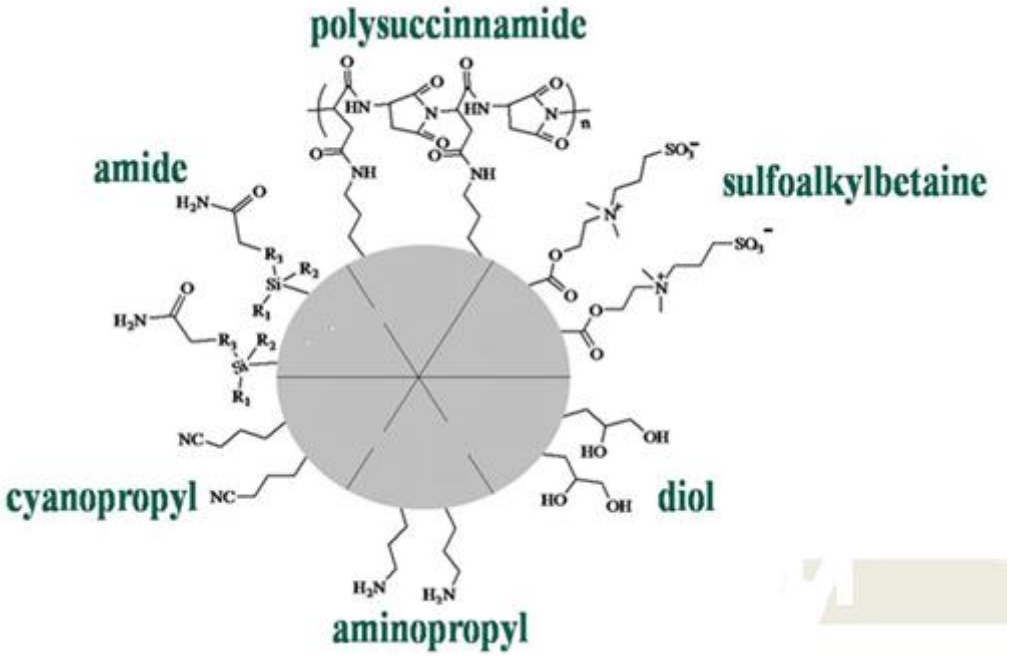


Figure 5.2. Stationary phases in HILIC, adapted with permission from Nguyen and Schug [116]

portion of the mobile phase, ion exchange / electrostatic interactions between the analyte ionized and the stationary phase, and adsorption (figure 5.3). It is often difficult to predict the predominant retention mechanism in HILIC. In order to know the predominant retention factor for the analytes studied and predict retention and separation in various stationary phases, it is common to utilize theoretical and empirical models during method development. For example, in case of aminoglycosides, when the ionic strength of mobile phase increases, the ion exchange decreases. When the pH of the mobile phase decreases, silanol interactions decrease thereby leaving the most dominant mechanism to be partitioning.

Aminoglycosides being highly polar, it is difficult to include them in multifamily analytical methods as the other chemical families vary considerably in their chemical properties. In that sense, a method to analyze only aminoglycosides is reasonable and the objective of this work was to

- Screen different HILIC stationary phases for chromatographic separation of aminoglycosides (Article II)

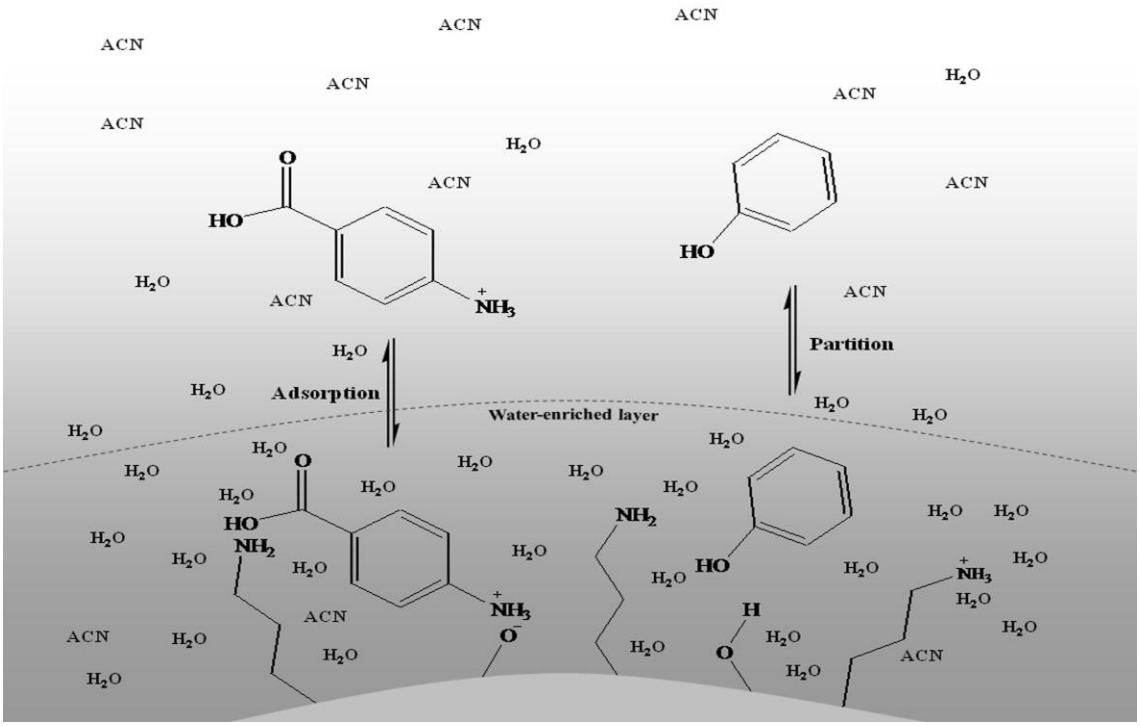


Figure 5.3. Multimodal retention mechanisms between a HILIC stationary phase and mobile phase. Reproduced with permission from Nguyen and Schug [116].

- Optimize mobile phase conditions to retain and separate aminoglycosides (Article II)
- Develop and validate analytical methodologies for analysis of ten aminoglycosides in animal kidney samples and honey samples (Article III)

The results of the work are reported in two scientific articles as below:

5.2. ARTICLE II

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Received October 3, 2011
Revised November 24, 2011
Accepted November 24, 2011

Research Article

Hydrophilic interaction chromatography for the analysis of aminoglycosides

The effect of mobile-phase constituents (pH and ionic strength) and chromatographic behaviour of ten aminoglycosides (streptomycin, dihydrostreptomycin, spectinomycin, apramycin, paramomycin, kanamycin A, gentamycin C1, gentamycin C2/C2a, gentamycin C1a and neomycin) in the bare silica, amino, amide and zwitterionic phases of hydrophilic interaction chromatography (HILIC) were studied systematically. Among the stationary phases studied, the zwitterionic phase provided the best separation of aminoglycosides. The effect of pH, ionic concentration and column temperature on retention time, peak shape and sensitivity was studied using a central composite design. pH affected sensitivity of the detection of analytes but not the retention time. High ionic strength in the mobile phase was necessary to control the ionic interactions between ionised aminoglycosides and the hydrophilic phase, thereby influencing peak shape and retention time. Column temperature affected sensitivity of the detection but not the retention time. During method development, crosstalk between the MS/MS channels of the analytes was observed and resolved.

Keywords: Aminoglycosides / Central composite design / HILIC
DOI 10.1002/jssc.201100860

1 Introduction

Hydrophilic interaction chromatography (HILIC) [1] is a variant of normal-phase chromatography that uses water as a strong eluent and water-miscible organic solvents like acetonitrile as organic components of the mobile phase. The ability of HILIC to retain highly polar compounds makes it complementary to the reversed-phase mode and explains its increased use in recent years [2–3]. The use of HILIC to analyse polar contaminants in food and environmental analysis is steadily increasing [4]. The retention mechanism in HILIC is multimodal and is a combination of adsorption, ionic/electrostatic interactions and liquid–liquid partitioning of analytes between the mobile phase and the water layer formed on the stationary phase. The predominant retention mechanism depends on the type of stationary phase chosen and the functional groups of the analytes [5]. In HILIC, apart from gradient composition and flow rate, factors such as pH, ionic concentration of the mobile phase and column temperature influence selectivity and sensitivity and need to be optimised during method development. Given the multimodal retention mechanism in HILIC, method development is often complex and a systematic approach is necessary [6].

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In the last ten years, much research has been conducted to characterise the stationary phases used in HILIC and study the effect of mobile-phase composition and temperature on the retention and selectivity of compound sets used as model analytes. Most of this work has been published since 2006 either in review papers or in special issues [5, 7–14]. Despite the great efforts carried out, conclusions from these studies tend to be general because of the complexity of HILIC systems, thus highlighting the need to experimentally assess the behaviour of each group of specific analytes. Regarding detection, some authors have demonstrated the suitability of electrospray ionisation (ESI)-MS in conjunction with HILIC mode separations [5, 8, 15]. This is attributed to the enhanced desolvation and ionisation efficiencies of the ESI source caused by the acetonitrile-rich mobile phase used in HILIC.

Aminoglycosides belong to a group of antibiotics used to treat Gram-negative bacterial infections. They are also used as growth promoters in food-producing animals, although this practice is currently banned in the European Union (EU). Aminoglycosides are basic and highly polar compounds, containing several amino groups with different pK_a values. The difference in the pK_a values is due to the steric effects and hydrogen bonds between the amino groups and neighbouring hydroxyl groups on the sugar rings [16]. The pK_a values of the aminoglycosides included in this study are listed in Supporting Information Table S1.

Aminoglycosides are analysed in eggs, milk, tissues and fluids of food-producing animals [17], as well as in human serum [18] and urine [19] for control and monitoring

reasons. The chromatographic separation of aminoglycosides can be performed with reverse-phase columns, using ion-pairing agents [19–29] or derivatisation agents [30] to achieve suitable retention. However, when MS is used as the detection technique, ion-pairing reagents can cause ion suppression and contaminate the instrument [31]. Recently, HILIC has been receiving more attention and several methods based on this technique have been proposed [18, 31–35].

Here, the behaviour of different stationary phases and mobile-phase constituents in the separation of aminoglycosides were studied, and a method development based on an experimental design is reported.

2 Materials and methods

2.1 Chemicals and solutions

Apramycin sulphate, dihydrostreptomycin sesquisulphate and amikacin disulphate (internal standard) were obtained from Sigma-Aldrich (Buchs, Switzerland). Paromomycin sulphate, spectinomycin dihydrochloride pentahydrate and streptomycin sulphate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Kanamycin A disulphate dihydrate, neomycin trisulphate hydrate and gentamycin sulphate were obtained from Sigma-Aldrich (Seelze, Germany). Gentamycin consists of a mixture of four components, gentamycin C1, C1a, C2 and C2a, where gentamycin C1, C2 and C2a are the major components. Gentamycin C2 and C2a are stereoisomers. Kanamycin contains kanamycin A as the major component and kanamycin B and C as minor components. Neomycin contains neomycin A, B and C. Neomycin B and C are stereoisomers. Individual stock standard solutions of 1 mg/mL were prepared with 10% methanol in water and stored at $-20^{\circ}C$ for up to 1 year.

Accell Plus CM SPE cartridges, 6 mL × 500 mg, were obtained from Waters (Milford, MA, USA).

Formic acid (89–91%) and ammonium acetate of analytical grade were obtained from Merck (Darmstadt, Germany). Ammonium formate of analytical grade and acetonitrile (LC grade) were obtained from Panreac Qumica SA (Barcelona, Spain). Double deionised water Milli Q (Millipore, Molsheim, France) of 18.2 MΩ/cm was used.

2.2 LC/MS/MS

Six chromatographic columns with different stationary phases were used: bare silica (Acquity BEH HILIC, 2.1 × 100 mm, 1.7 µm, Waters, Milford, USA; Atlantis HILIC, 3 × 100 mm, 3 µm, Waters; Kinetex HILIC, 3 × 150 mm, 2.6 µm, Phenomenex, Torrance, CA, USA), amino (Luna NH_2, 4.6 × 150 mm, 3 µm, Phenomenex), amide (TSK gel Amide 80, 2 × 150 mm, 3 µm, Tosoh, Tokyo, Japan) and zwitterionic (ZIC®–HILIC, 2.1 × 150 mm, 3.5 µm, SeQuant AB, Umea, Sweden).

A Waters Acquity UPLC system (Manchester, UK) coupled to a Quattro Premier triple-quadrupole mass spectrometer from Micromass (Manchester, UK) equipped with an ESI source was used. The ESI source was operated in the positive mode with the following conditions: capillary voltage of +3.5 kV; source block and desolvation temperatures of 150 and 450°C, respectively; desolvation and nebuliser gas (nitrogen) flow rates of 996 L/h and 116 L/h, respectively; and argon pressure in the collision cell of 4×10^{-3} mbar. The MRM method parameters are provided in Supporting Information Table S2.

2.3 Data analysis

The experimental design was carried out using the SIRIUS software, kindly provided by Pattern Recognition System AS (Bergen, Norway).

2.4 Procedures

2.4.1 Screening of stationary phases

To study the separation capability of the stationary phases and the influence of pH and ionic concentration, five experiments in triplicates were carried out by changing pH and ionic concentration of the aqueous mobile phase (mobile phase A), as shown in Supporting Information Table S3. Mobile phase B was kept constant as 0.2% formic acid in acetonitrile. The typical operating flow rate, which depends on column and particle dimensions, and the gradient were optimised for each stationary phase (Supporting Information Table S4).

2.4.2 Optimisation of mobile-phase constituents and column temperature with ZIC® HILIC

After choosing the optimal stationary phase, ZIC® HILIC, the effect of pH, ionic concentration and column temperature on retention time and sensitivity (peak area) were studied using a central composite design. pH and ionic concentration were varied only in mobile phase A. A pH 5.5 buffer was prepared with ammonium acetate and for other pH values, ammonium formate was used. Supporting Information Table S5 shows the three mobile-phase factors at five levels, whereas Table 1 shows the experimental conditions of the central composite design. Each experiment was run in triplicates.

2.4.3 Extraction of aminoglycosides from honey samples

Samples of 3 g of honey were introduced into a polypropylene tube, spiked with the internal standard and allowed to stand for 10 min. Twenty milliliters of double deionised water was added to the spiked honey and dissolved by

Table 1. Central composite design of the experiments

Run order	pH	Ionic concentration (mM)	Column temperature (°C)
1	3	75	20
2	3.5	125	30
3	4.5	75	20
4	3	175	20
5	3	75	40
6	3.5	125	13
7	3.5	125	46
8	4.5	75	40
9	3	175	40
10	2.8	125	30
11	3.5	125	30
12	4.5	175	40
13	5.5	125	30
14	3.5	41	30
15	3.5	125	30
16	4.5	175	20
17	3.5	209	30
18	4.5	175	40

vortexing for 30 s. The dissolved sample was loaded onto a weak cation-exchange SPE cartridge conditioned with 3 mL of MeOH and 3 mL of water. The cartridge was washed with 3 mL of water and dried for 10 min. The analytes were eluted with 2 mL of 175 mM ammonium formate (pH 3). The eluate was vortexed, filtered and injected into the LC-MS/MS.

3 Results and discussion

3.1 Need for chromatographic separation (crosstalk)

Crosstalk effect occurs in low-resolution mass spectrometry, when several mass transitions with identical precursor or product ions in coeluting peaks are acquired [36]. For example, paromomycin (616.5) and neomycin (615.5) differ by 1 Da in their precursor ions and also have identical product ions (163.0). Other analytes also have identical product ions, such as neomycin (161.0), gentamycin C1a (160.1) and gentamycin C2/C2a (160.0). When all these analytes coelute in the LC, the signals of identical precursor ions interfere with each other and also it is difficult to completely empty the product ions formed in the collision cell within the very short time between each transition (interchannel delay) and each scan (interscan delay). This leads to inaccuracy in the quantitation of analytes. To ensure accurate measurements, it is necessary to have complete chromatographic separation of the aminoglycosides. Tobramycin was initially chosen as an internal standard, but there was an increase in its signal with increased analyte concentrations due to crosstalk between some analyte transitions and tobramycin transitions. Therefore, amikacin was chosen as the internal standard, which does not share identical precursor or product ions with the analytes.

3.2 Screening of the stationary phase

Screening experiments were carried out to find the working range of the mobile-phase conditions (pH and ionic concentration) in four HILIC phases and choose the stationary phase that provides the optimal chromatographic separation.

Stationary phases with different charge states, such as bare silica (negative), aminopropyl (positive), amide (neutral) and ZIC® HILIC (zwitterionic), were chosen for the screening experiments. For bare silica, three columns of different silica types and particle sizes were assessed: Acquity BEH HILIC with ethylene-bridged hybrid particles (1.7 μm), Atlantis HILIC (3 μm) and Kinetex HILIC (2.6 μm) with core-shell characteristics. Acquity BEH HILIC did not retain any of the aminoglycosides, while Atlantis HILIC gave similar relative retentivity and selectivity behaviour as Kinetex HILIC, but Kinetex HILIC provided better peak shapes. Peak asymmetry was calculated as b/a, where b is the width of the back half of the peak and a is the width of the front half of the peak measured at 5% of peak height. The peak asymmetry of the analytes in Kinetex HILIC were between 0.79 and 1.91 and in Atlantis HILIC between 0.72 and 7. Therefore, Kinetex HILIC was chosen as a representative of bare silica for the screening study.

In HILIC, pH and ionic concentration of the mobile phase affect retention and separation selectivity. As the analytes are ionisable, ionic interactions can vary according to the charge state of the stationary phase and the mobile-phase conditions. With increasing ionic concentration, ionic interaction between the stationary phase and analytes are disrupted and liquid–liquid partitioning plays a dominant role [37]. Moreover, a low-pH mobile phase can neutralise free silanols and prevent interactions between analytes and silanol groups, making the partitioning and the interactions between ionised analytes and the bonded phase dominant. Regarding detection, basic pH gave poor sensitivity for most of the analytes since aminoglycosides are basic compounds. As most of the pK_a values of aminoglycosides are above 7, it is necessary to work at acidic pH to ionise aminoglycosides and therefore have good sensitivity in ESI-MS. Therefore, two acidic pH values (3 and 4.5), a low and a high ionic concentration (5 and 175 mM) and 1% formic acid were chosen as mobile phase A to screen stationary phases.

At the low ionic concentration, in all the phases except Kinetex HILIC, most of the aminoglycosides were highly retained and showed bad peak shapes due to the strong ionic interactions and partitioning. In Kinetex HILIC (bare silica), even at the lower ionic concentration, the analytes were less retained and had relatively good peak shapes (b/a – 1.09 to 4.96), which could be because of the core-shell particle characteristics where the partitioning of the analytes between the stationary phase and mobile phase is faster.

At the high ionic concentration in all the phases, aminoglycosides were less retained and the peak shapes were improved. For instance, in Kinetex HILIC, peak asymmetries were between 1.01 and 2.99. On the other hand, when 1% formic acid was used as the mobile phase A with the bare silica, amide and amino phases, sharp and symmetrical peaks were observed due to minimised silanol interactions. In each screening experiment, the initial gradient was altered to improve the separation of analytes with no success, indicating that the separation selectivity of aminoglycosides is mainly dictated by the stationary phase rather than changes in the gradient conditions. In all the columns, pH 4.5 gave considerably higher sensitivity than pH 3. The retention times of aminoglycosides in the four stationary phases at pH 4.5 with 125 mM ammonium formate as the mobile phase A is shown in Fig. 1. ZIC® HILIC gave better separation of aminoglycosides than the other stationary phases. For analysing aminoglycosides, it was important to chromatographically separate the analytes for avoiding crosstalk in low-resolution mass spectrometry.

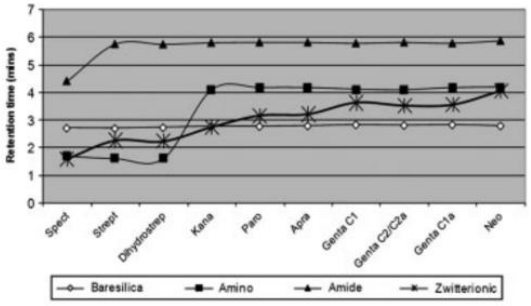

Figure 1. Retention time of aminoglycosides in the bare silica, amino, amide and zwitterionic phases with the conditions of the screening experiment number 4.

Therefore, the optimal working conditions are ZIC® HILIC for better separation, acidic pH for good sensitivity of the analytes and high ionic concentration for good peak shape and retention.

3.3 Optimisation of pH, mobile-phase and ionic concentration with ZIC® HILIC

After choosing ZIC® HILIC for its better separation of analytes, the effect of pH, ionic concentration and column temperature (3 factors) on retention time and sensitivity (2 responses) was evaluated using a central composite experimental design. Supporting Information Table S5 shows the values of the factors studied and Table 1 gives the specific conditions of the eighteen experiments carried out. In experiment no. 13, no responses were obtained for most of the analytes at pH 5.5. Hence, experiment 13 was not considered for evaluating the model.

The response surface plots of the model show the retention time (Fig. 2A) and peak sensitivity (Supporting Information Fig. S1a) against the three factors studied for gentamycin C1. The correlation plot between predicted and measured responses (retention time and peak area) is shown in Fig. 2B and Supporting Information Fig. S1b. An R^2 value of 0.935 was observed for the model predicting the retention time and 0.780 for the model predicting the peak area. For spectinomycin and neomycin, R^2 values of the models ranged from 0.634 to 0.925.

With the increase in ionic concentration, retention time decreased as the ion exchange of analytes with the stationary phase decreased. The effect of pH on retention time was less important than that of ionic concentration, while the effect of temperature was negligible (Fig. 2A).

pH and temperature significantly influenced the sensitivity of analytes, while the ionic concentration had

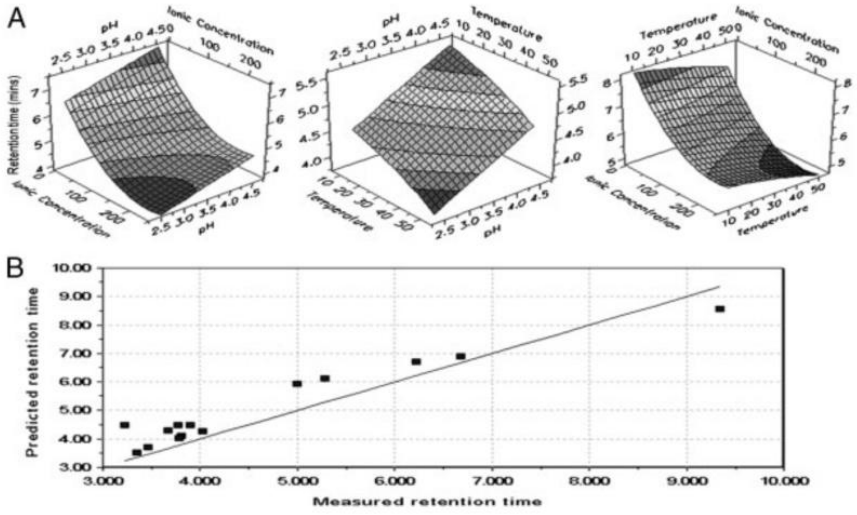

Figure 2. 3D contour plots of the model showing the effect of pH, temperature and ionic concentration on retention time for gentamycin C1 (A). Correlation plot showing the correlation between predicted and measured retention time (B).

negligible effect (Supporting Information Fig. S1a). To confirm the optimal conditions, univariate experiments were carried out to confirm the optimal conditions. Supporting Information Figs. S2 and S3 summarise the results of univariate experiments on retention time and peak area for all the analytes, respectively. The sensitivity of the analytes increased until 40°C for all the analytes and then decreased for some analytes (Supporting Information Fig. S3a). Therefore, 40°C was decided to be the optimal column temperature. pH 4.5 gave high sensitivity for most of the aminoglycosides (Supporting Information Fig. S3b), while 175 mM ammonium formate was deemed to be optimal as it provided good peak shapes (Fig. S4 in the case of gentamycin C1). The asymmetry of analyte peaks in optimal mobile-phase conditions ranged between 0.82 and 1.73.

LOD, LOQ, linearity range, retention time and peak area precision of the optimised LC method are presented in Table 2. LOD and LOQ values were estimated on the s/n ratio, where LOD corresponds to the concentration of

Table 2. LOD, LOQ and % RSD for retention time and peak area ($n = 10$) of standards and working range of the method for the aminoglycosides studied

Analyte	LOD (μg/L)	LOQ (μg/L)	% RSD for retention time	% RSD for peak area	Linearity range assessed (μg/L)
Streptomycin	3	10	0.00	3	20–2000
Dihydrostreptomycin	3	9	0.14	2	20–2000
Spectinomycin	2	6	0.31	3	70–2000
Kanamycin A	8	27	0.16	3	70–2000
Apramycin	12	40	0.21	2	70–2000
Paromomycin	14	48	0.15	3	70–2000
Gentamycin C1	18	60	0.23	2	20–2000
Gentamycin C1a	31	104	0.23	1	70–2000
Gentamycin C2/C2a	21	68	0.20	3	70–2000
Neomycin	46	154	0.29	3	200–2000

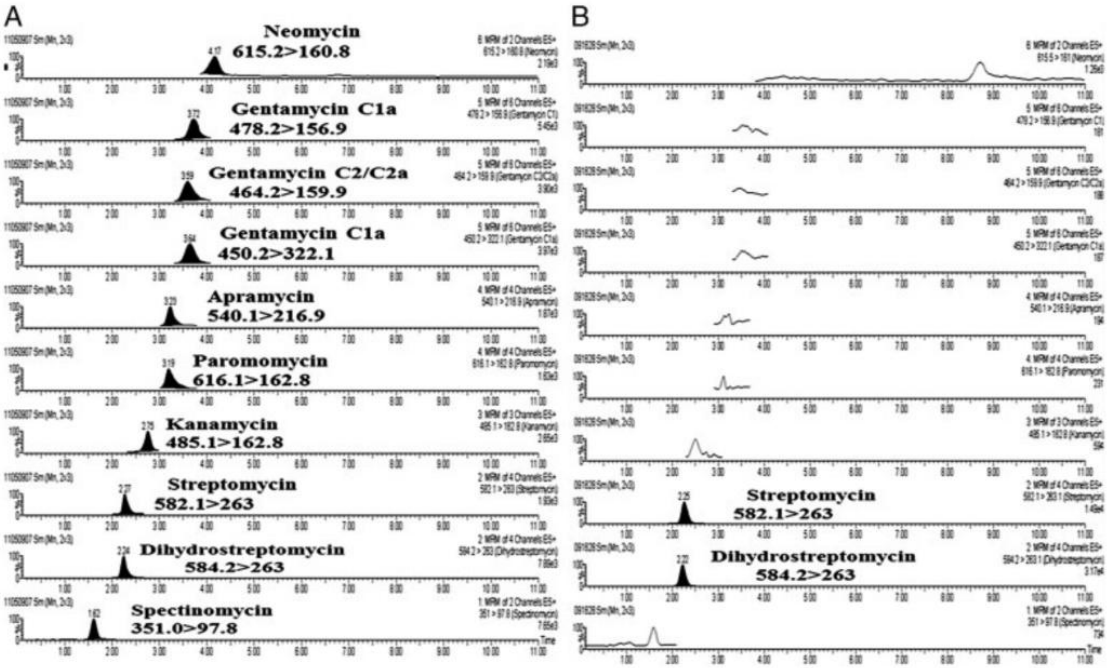

Figure 3. Chromatogram of aminoglycosides in spiked honey sample at LOQ levels – 20 μg/kg (streptomycin, spectinomycin and dihydrostreptomycin), 40 μg/kg (gentamycin C1, gentamycin C1a, gentamycin C2/C2a), 70 μg/kg (apramycin, paromomycin and kanamycin A) and 150 μg/kg (neomycin) with ZIC® HILIC using the optimised mobile phase conditions (A). Chromatogram of aminoglycosides in an interlaboratory honey sample with streptomycin at 40 μg/kg and dihydrostreptomycin at 43.3 μg/kg (B).

analyte at which the s/n ratio is 3 and LOQ corresponds to the concentration of the analyte at which the s/n ratio is 10.

3.4 Injection solvent

Acetonitrile, water, mobile phase A and a mobile phase with the initial composition were studied as the injection solvent. Mobile phase A gave the best peak shapes and sensitivity for all the analytes. Acetonitrile, in particular as an injection solvent, gave poor sensitivity for most of the analytes, possibly due to the limited solubility of aminoglycosides in acetonitrile.

3.5 Analysis of honey samples

The optimized method has been applied for the analysis of aminoglycoside residues in honey. An MRL of 40 μg/kg in honey for streptomycin has been recommended by community reference laboratory, Fougeres, and for other aminoglycosides no MRLs have been laid. Extraction recoveries were assessed by spiking blank samples at 100 μg/kg for streptomycin and 200 μg/kg for the other analytes. Extraction recoveries ranged between 68 and 112%. Matrix-matched standards and internal standard were used for quantification. The linearity was verified ($R^2 > 0.99$) from 20 to 150 μg/kg for streptomycin and from LOQ levels to 500 μg/kg for other analytes. LOQs in sample are 20 μg/kg (streptomycin, spectinomycin and dihydrostreptomycin), 40 μg/kg (gentamycin C1, gentamycin C1a, gentamycin C2/C2a), 70 μg/kg (apramycin, paromomycin and kanamycin A) and 150 μg/kg (neomycin). Intermediate precision ($n = 18$) at LOQ levels studied in three different days ranged between 12 and 27% for all the analytes studied. The precision at three higher levels – 40, 80 and 120 μg/kg for streptomycin, 150, 300 and 450 μg/kg for other aminoglycosides, ranged between 11 and 19%. Figure 3A displays a chromatogram of aminoglycosides in blank honey sample spiked at LOQ levels and Fig. 3B displays a chromatogram of aminoglycosides in an interlaboratory honey sample (FAPAS).

4 Concluding remarks

The interaction between aminoglycosides, different HILIC phases (bare silica, aminopropyl, amide and zwitterionic stationary phases) and mobile-phase constituents (pH and ionic concentration) was assessed. Column selectivity and retention of aminoglycosides depended greatly on the HILIC phase chosen, followed by ionic strength in the mobile phase. pH did not affect selectivity, but influenced sensitivity of the analytes. Formic (1%) acid as the mobile phase gave good peak shapes in bare silica, amide and amino phases. In terms of chromatographic separation, ZIC® HILIC provided better resolution of peaks than the other phases, which was necessary to avoid the crosstalk between analyte signals. Ammonium formate (175 mM) at pH 4.5 was the optimal mobile phase for achieving good sensitivity and satisfactory retention of all the aminoglycosides. However, from our experience, if only streptomycin, dihydrostreptomycin and spectinomycin have to be analysed, high ionic strength in the mobile phase is not necessary. Considering the complexity of optimising multiresidue methods based on HILIC, the use of experimental designs (a multivariate approach) allows optimisation of the conditions with limited experiments (<30); a univariate approach requires more than a hundred experiments. Moreover, the use of response surface enables visualisation of the effects of factors on the chosen responses (retention time and peak area). The developed method has been applied for the analysis of aminoglycoside residues in honey.

This study was supported by the Spanish Ministerio de Ciencia e Innovacion (Project AGL 2008-05578-C05-03). P. Kumar thanks the Generalitat de Catalunya for an FI grant (Grant Number 2010 FI-B 00676).

The authors have declared no conflicts of interest.

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Supporting Information

Figures

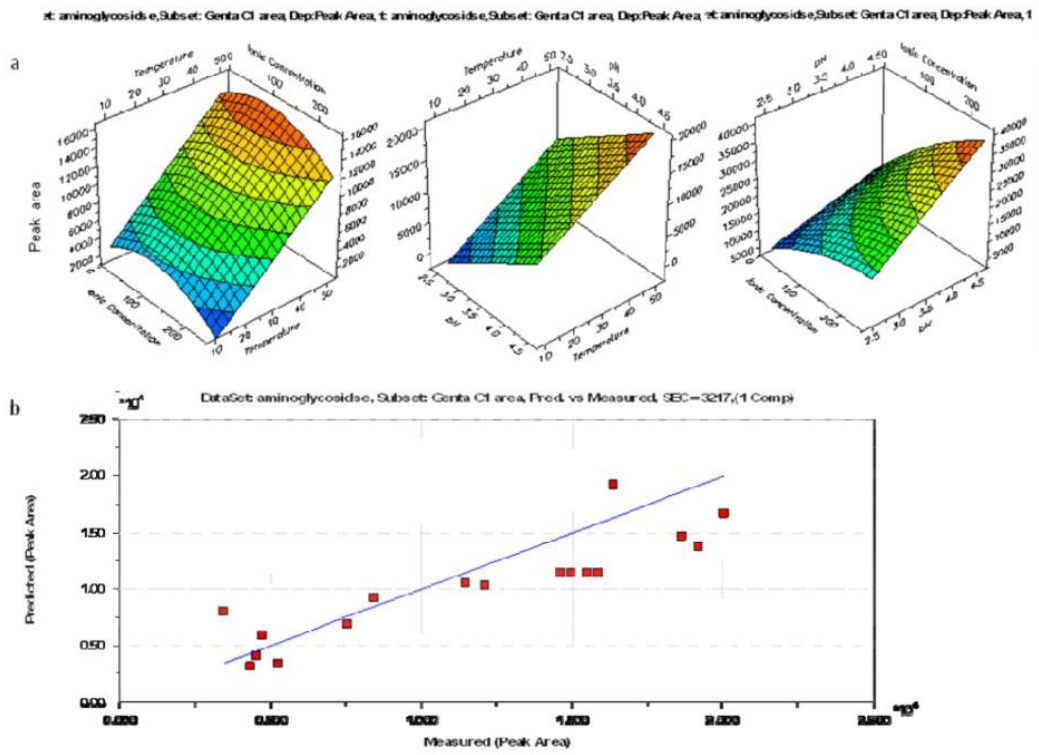
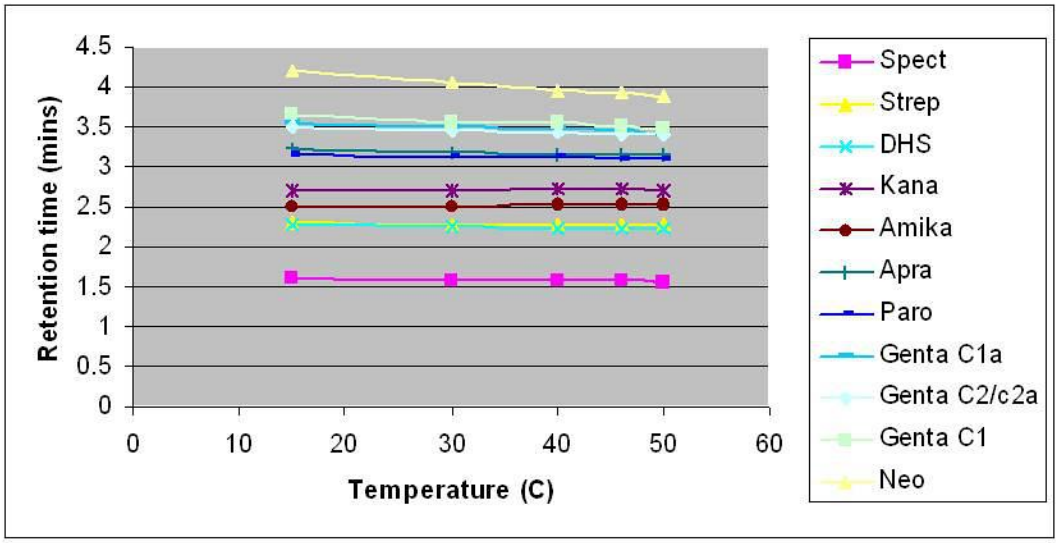


Figure S1. 3D contour plots of the model showing the effect of pH, temperature and ionic concentration on peak area for Gentamycin C1 (S1 a). Correlation plot showing the correlation between predicated and measured peak area (S1 b)

a



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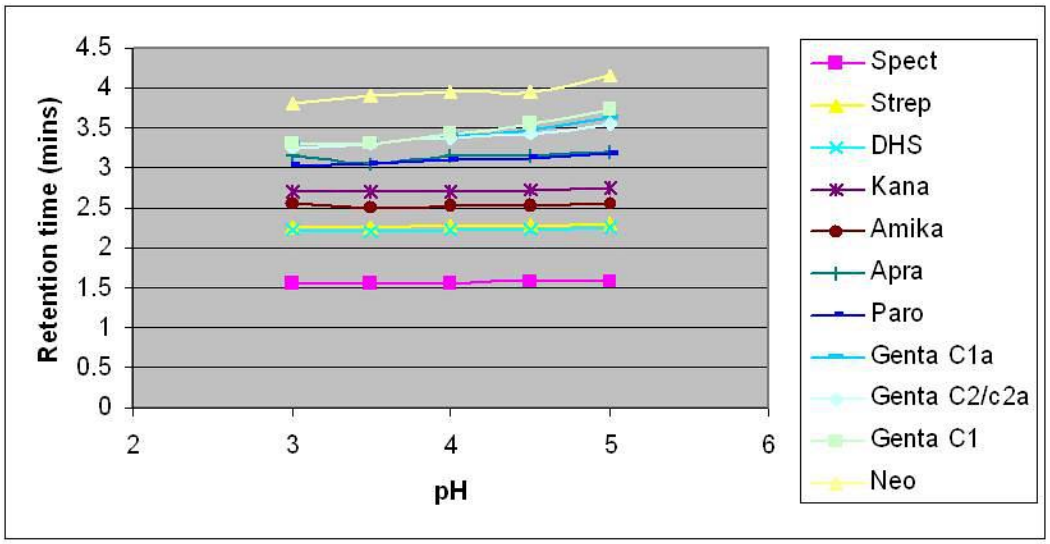

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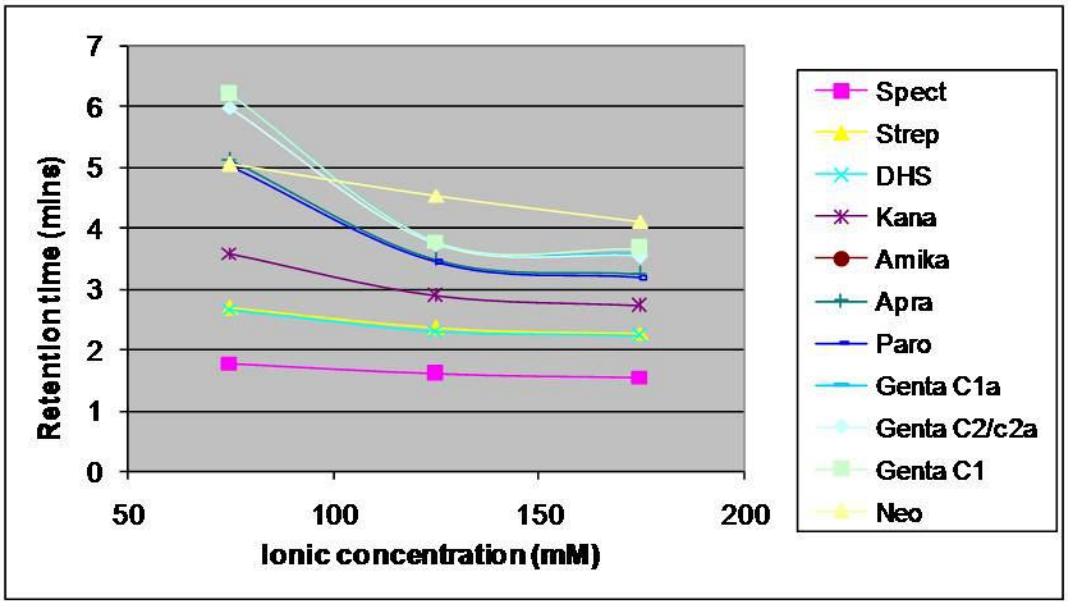
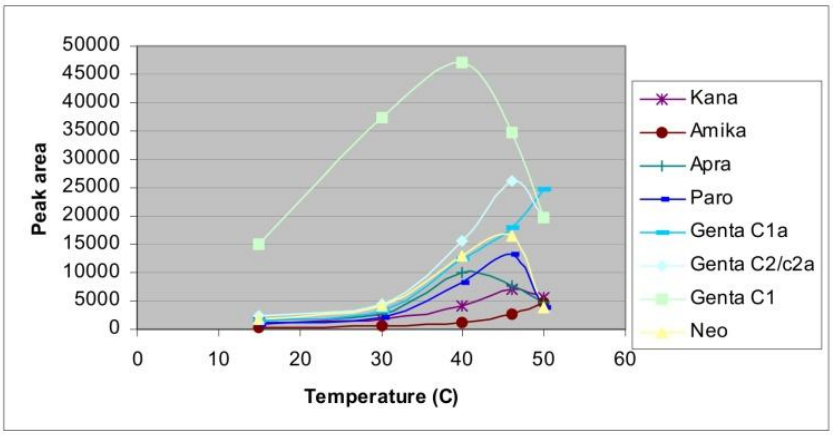


Figure S2. Univariate experiments with the ZIC® HILIC column. Temperature vs retention time (S2 a), pH vs retention time (S2b) and Ionic concentration vs rentention time (S2c)

a



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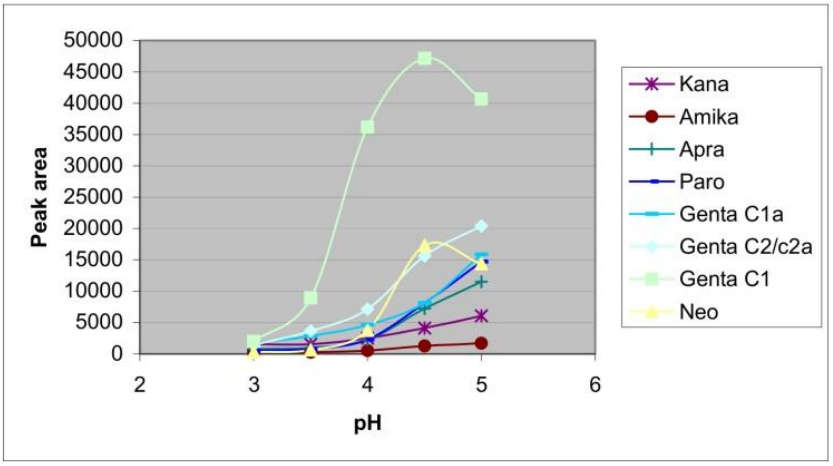

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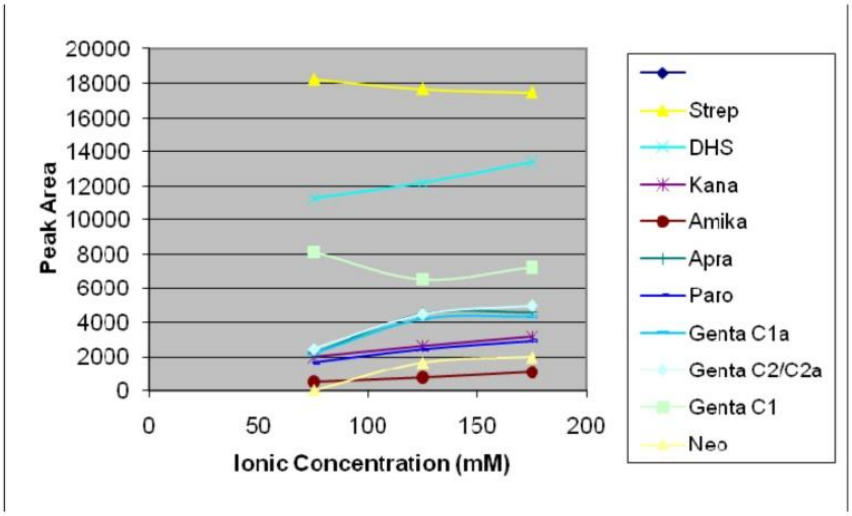

Figure S3. Univariate experiments with the ZIC HILIC column. Temperature vs peak area (S3a), pH vs peak area (S3b) and ionic concentration vs peak area (S3c)

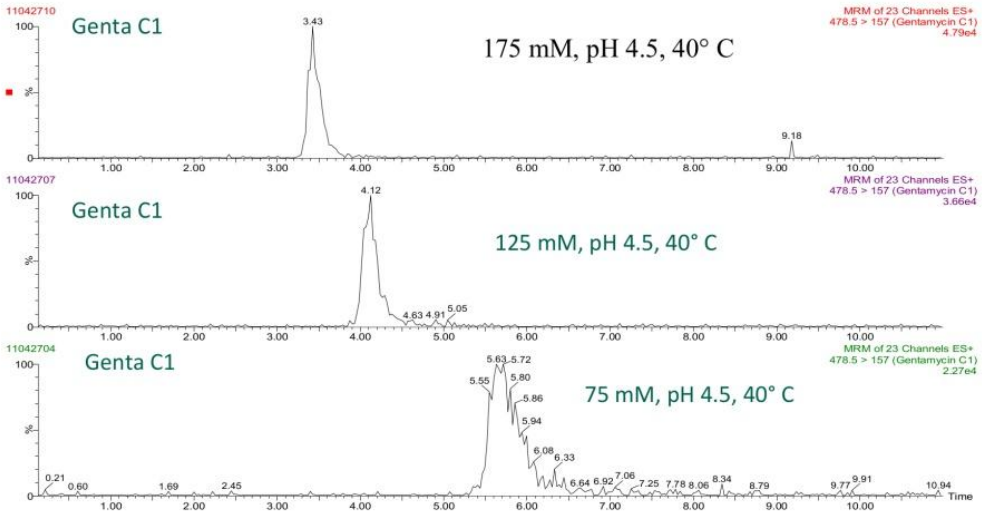

Figure S4. Effect of ionic concentration on the peak shape of gentamycin C1

Tables

Table S1. pK_a values of the aminoglycosides studied.

Aminoglycosides	pK_a*
Streptomycin	8.7 [1]
Dihydrostreptomycin	8.8 [2]
Spectinomycin	6.78 & 8.8 [3]
Apramycin	5.4 to 8.5 [4]
Kanamycin	7.2 [1]
Neomycin	5.7 to 8.8 [5]
Paramomycin	5.9 to 8.9 [6]
Gentamycin	8.2 [2]

*where numbers in [ ] indicate the references

[1] Nishi, H., Nakamura, K., Nakai, H., Sato, T., *Chromatographia* 1996, *43*, 426-430.
[2] Aminoglycosides (Veterinary - Systemic), The United States Pharmacopeial Convention,2007,
[3] Wang, M., Hu, C., *Chromatographia* 2006, *63*, 255-260.
[4] McGlinchey, T. A., *Anal. Chim. Acta* 2008, *624*, 1-15.
[5] Clouet-d'Orval, B., Stage, T. K., Uhlenbeck, O. C., *Biochemistry* 1995, *34*, 11186-11190.
[6] Harry, G. B., *Profiles Drug Subst., Excipients, Relat. Methodol.*, Academic Press, London 2007.

Table S2. Multiple Reaction Monitoring (MRM) parameters for the aminoglycosides studied.

Analyte	Cone voltage (V)	Quantification trace (m/z)	Collision energy (eV)	Confirmation trace (m/z)	Collision energy (eV)
Spectinomycin	35	351.0>333.1	18	351.0>97.8	30
Streptomycin	55	582.1>263	30	582.1>221.2	40
Dihydrostreptomycin	55	584.2>263	30	584.2>246.0	40
Kanamycin A	25	485.1>162.8	30	485.1>324.1	15
Paromomycin	40	616.1>162.8	30	616.1>202.9	30
Apramycin	30	540.1>216.9	30	540.1>378.1	20
Neomycin	50	615.2>160.8	30	615.2>163	35
Gentamycin C1a	25	450.2>322.1	15	450.2>159.9	20
Gentamycin C2/C2a	20	464.2>159.9	25	464.2>322.1	10
Gentamycin C1	30	478.2>156.9	20	478.2>322.1	15
Amikacin (internal standard)	30	586.1>425	20		

Table S3. Mobile phase A conditions studied with four different stationary phases.

Experiments	pH	Ionic concentration (mM)
1	3	5
2	4.5	5
3	3	125
4	4.5	125
5	1% formic acid (pH 2.5)	

Table S4. Generic gradient conditions for the different stationary phases studied.

Stationary phase	Flow rate (ml/min)	Gradient	
		Time	%A
Zwitterionic (sulphoalkylbetaine)	0.5	0 2 6	40 85 85
Amide (-RCONH)	0.15	0 3 6	45 85 45
Aminopropyl ($-RNH_2$)	0.6	0 2.5 5 6	25 95 95 25
Bare silica (Si-OH)	0.4	0 1.5 3 4	15 95 95 15

Table S5. Factors and levels studied in the experimental design

	-1.682	**-1**	**0**	**1**	**1.682**
pH	2.8	3	3.5	4.5	5.5
Ionic concentration (mM)	41	75	125	175	209
Column temperature (^oC)	13	20	30	40	46

5.3. ARTICLE III

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Received April 3, 2012
Revised June 29, 2012
Accepted June 29, 2012

Research Article

Determination of aminoglycoside residues in kidney and honey samples by hydrophilic interaction chromatography-tandem mass spectrometry

Two methods based on liquid chromatography–tandem mass spectrometry were developed for the determination of ten aminoglycosides (streptomycin, dihydrostreptomycin, spectinomycin, apramycin, paromomycin, kanamycin A, gentamycin C1, gentamycin C2/C2a, gentamycin C1a, and neomycin B) in kidney samples from food-producing animals and in honey samples. The methods involved extraction with an aqueous solution (for the kidney samples) or sample dissolution in water (for the honey samples), solid-phase extraction with a weak cation exchange cartridge and injection of the eluate into a liquid chromatography–tandem mass spectrometry system. A zwitterionic hydrophilic interaction chromatography column was used for separation of aminoglycosides and a triple quadrupole mass analyzer was used for detection. The methods were validated according to Decision 2002/657/EC. The limits of quantitation ranged from 2 to 125 μg/kg in honey and 25 to 264 μg/kg in the kidney samples. Interday precision (RSD%) ranged from 6 to 26% in honey and 2 to 21% in kidney. Trueness, expressed as the percentage of error, ranged from 7 to 20% in honey and 1 to 11% in kidney.

Keywords: Aminoglycosides / Food of animal origin / Hydrophilic interaction chromatography / Tandem mass spectrometry / Weak cation exchange SPE
DOI 10.1002/jssc.201200344

1 Introduction

Aminoglycosides are bactericidal antibiotics that are active against Gram-negative bacteria and some Gram-positive bacteria. They are used in animals for therapeutic purposes rather than for metaphylaxis or prophylaxis. Gentamycin is used for Gram-negative sepsis. Streptomycin and neomycin B are used for treating bacterial enteritis in calves, as well as bovine mastitis. Aminoglycosides are large polar molecules that do not readily enter cells and their elimination is entirely via the kidney [1]. All of them show ototoxicity and nephrotoxicity. Streptomycin is used to treat American foulbrood disease in honey bees [2] and fire blight on fruit trees, which is caused by the bacterium *Erwinia amylovora* and can lead to streptomycin residues in honey [3].

The toxicology and residue depletion data of aminoglycosides were evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and reviewed by the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) for the establishment of maximum residue limits (MRLs) to be adopted by the Codex Alimentarius Commission (CAC). The toxicology and recommended MRLs of all aminoglycosides in different matrices are included in monographs and archived on the JECFA website [www.fao.org/ag/agn/jecfa-vetdrugs/search.html]. In the European Union (EU), the MRLs for aminoglycosides in the kidney, muscle, fat, liver, milk, and eggs of different animal species are set by EC Regulation 37/2010 [4], in which the MRL values are lower than those set by the CAC. Supporting Information Table S1 shows the MRL values set by the EU and the CAC for aminoglycosides in kidney. No MRLs have been set for honey, except in the case of streptomycin, which has a recommended limit of 40 μg/kg set by the Community Reference Laboratory, Fougères (France) [www.rivm.nl/bibliotheek/digitaaldepot/crlguidance2007.pdf]. Since MRLs for aminoglycosides in honey have not been established, they must be considered as banned substances. Aminoglycosides are listed under Group B1 as antibacterial substances in the Council Directive 96/23/EC [5] and all antibiotics have been prohibited for the use of growth promotion in food-producing animals in the EU since 2006 [6].

From an analytical perspective, aminoglycosides are highly polar in nature and have multiple ionization sites, and

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Abbreviations: CAC, Codex Alimentarius Commission; **EU**, European Union; **HILIC**, hydrophilic interaction chromatography; **IPC**, ion-pairing chromatography; **MRL**, maximum residue limit; **WCX**, weak cation exchange

are therefore difficult to separate chromatographically. They are also difficult to detect due to lack of chromophores and to their poor MS responses. Moreover, it is difficult to include them in multifamily methods because their physicochemical properties differ from those of other veterinary drugs. The most recent multiresidue methods used for analyzing aminoglycosides are summarized in Supporting Information Table S2. The analysis of aminoglycosides by LC/MS is generally carried out using hydrophilic interaction chromatography (HILIC) [7–12] or ion-pairing chromatography (IPC) [13–26]; [www.fsis.usda.gov/PDF/CLG_AMG_2_03.pdf]. In a few cases, reverse-phase chromatography has been used after derivatization of analytes with phenylisocyanate [27, 28]. The use of IPC or HILIC is often questioned since IPC may cause ion suppression in mass spectrometer and also contaminate the whole LC-MS/MS system [9, 21]. Furthermore, a recent article by Kaufmann et al. points out that the HILIC method gives low sensitivity for late-eluting peaks [21].

In this study, two HILIC-based methods for analyzing ten aminoglycosides (spectinomycin, streptomycin, dihydrostreptomycin, kanamycin A, paromomycin, apramycin, neomycin B, gentamycin C1a, gentamycin C2/C2a, and gentamycin C1) in kidney and honey samples, based on a previously developed HILIC separation [29], were optimized and validated in accordance with Decision 2002/657/EC. In the previous paper, several columns based on bare silica, amino, amide, and zwitterionic phases were compared. After choosing the zwitterionic column, the effect of pH and ionic strength of the mobile phase as well as the column temperature on retention time, peak shape, and sensitivity was studied using a central composite design.

2 Materials and methods

2.1 Chemicals and solutions

Apramycin sulphate, dihydrostreptomycin sesquisulphate, and amikacin disulphate (internal standard) were obtained from Sigma-Aldrich (Buchs, Switzerland). Paromomycin sulphate, spectinomycin dihydrochloride pentahydrate, and streptomycin sulphate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Kanamycin A disulphate dihydrate, neomycin B trisulphate hydrate and gentamycin sulphate were obtained from Sigma-Aldrich (Seelze, Germany). Individual stock standard solutions of 1 mg mL^{-1} were prepared with 10% methanol in water and stored at –20°C for up to one year.

Gentamycin consists of a mixture of four components, gentamycin C1, C1a, C2, and C2a, (gentamycin C1, C2, and C2a being the major components). Gentamycin C1, C2, and C1a are closely related compounds with a common structure. Gentamycin C2 and C2a are stereoisomers. Kanamycin contains kanamycin A as the major component and kanamycin B and C as minor components. Kanamycin A and C are isomers and kanamycin B has different functional groups. Neomycin B contains neomycin A, B, and C. Neomycin B and C are stereoisomers. Supporting Information Fig. S1 shows the structures and isomers of gentamycin, kanamycin, and neomycin.

Analytical-grade formic acid (89–91%) was obtained from Merck (Darmstadt, Germany). Analytical-grade ammonium formate and LC-grade acetonitrile were obtained from Panreac Química S.L.U. (Barcelona, Spain). All other reagents used were of analytical grade. Double-deionized water (Milli Q, Millipore, Molsheim, France) of 18.2 MΩ cm^{-1} was used.

2.2 Apparatus

Accell Plus CM SPE cartridges of 6 mL × 500 mg (53.7 μm) with silica support were obtained from Waters (Milford, MA, USA). Bakerbond weak cation exchange (WCX) of 6 mL × 500 mg (47–61 μm) obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA), Strata X CW of 6 mL × 500 mg (33 μm) obtained from Phenomenex (Torrance, CA, USA), Sampliq WCX of 6 mL × 150 mg (60 μm) obtained from Agilent Technologies (Santa Clara, CA, USA), and Oasis MCX of 6 mL × 500 mg (60 μm) obtained from Waters, all with polymeric support, were also used. A refrigerated centrifuge from Hettich (Tuttlingen, Germany) and a 20-position SPE manifold from Waters were used.

2.3 LC-MS/MS conditions

A Zwitterionic column (ZIC®–HILIC, 2.1 × 150 mm, 3.5 μm, SeQuant AB, Umea, Sweden) was used. Mobile phase A consisted of 175 mM ammonium formate (pH 4.5) and mobile phase B consisted of 0.2% formic acid in acetonitrile. A linear gradient of (*t*, %A): (0, 40); (2, 85); (6, 85); (6.5, 40); (10, 40) was used. The mobile-phase flow rate used was 0.5 mL/min and the injection volume was 5 μL. An Acquity ultra performance liquid chromatography system coupled to a Quattro Premier triple-quadrupole mass spectrometer equipped with an electrospray ionization source from Waters (Manchester, UK) was used. The electrospray ionization source was operated in positive mode with the following conditions: capillary voltage of 3.5 kV; source block and desolvation temperatures of 150 and 450°C, respectively; desolvation and nebulizer gas (nitrogen) flow rates of 996 and 116 L h^{-1}, respectively; and argon pressure in the collision cell of 4×10^{-3} mbar. The multiple reaction monitoring method parameters are provided in Table 2. Instrument control and data processing were carried out using Masslynx 4.1 software.

2.4 Sample extraction and clean up

2.4.1 Honey

A 3 g honey sample was weighed into a 50 mL polypropylene tube, spiked with the internal standard (amikacin) and

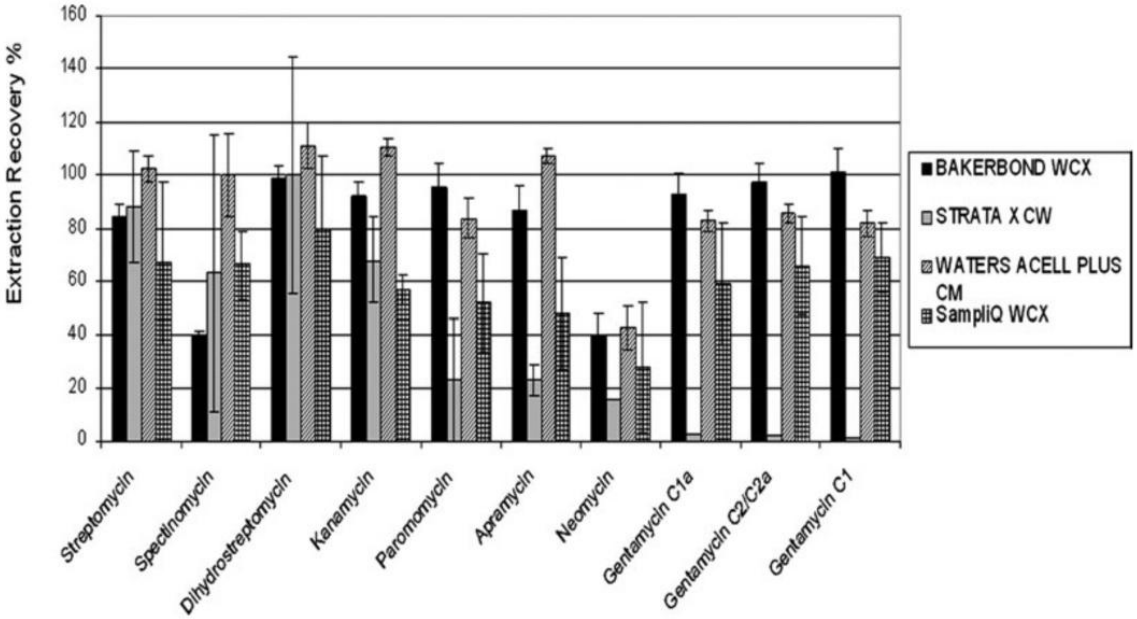

Figure 1. Extraction recoveries of aminoglycosides with different WCX cartridges ($n = 5$ replicates).

allowed to stand for 30 min. Twenty milliliters of double-deionized water was added to the spiked honey and dissolved by vortexing for 30 s. The dissolved sample was loaded onto a WCX SPE cartridge (Accell Plus CM), previously conditioned with 3 mL of methanol and 3 mL of water. The cartridge was washed with 3 mL of water and dried for 10 min with vacuum. The analytes were eluted with 2 mL of 175 mM ammonium formate (pH 3). The eluate was vortexed, filtered, and injected into the LC-MS/MS system.

2.4.2 Kidney

A 3 g sample of minced kidney tissue was weighed in a 50 mL polypropylene centrifuge tube, spiked with the internal standard (amikacin), and allowed to stand for 30 min. Twenty milliliters of extraction solution (10 mM ammonium acetate/0.4 mM EDTA/1% NaCl/2% TCA) were added to the sample and homogenized in a vortex for 30 s. The sample was shaken in a mechanical shaker for 10 min and centrifuged at 3000 rpm for 10 min. The supernatant was transferred to another tube and the pH was adjusted to 6.5 with NaOH (20%) or HCl (1 M) solutions. This step was followed by centrifugation for 10 min at 3000 rpm. The supernatant was loaded onto a WCX SPE cartridge (Accell Plus CM) previously conditioned with 3 mL of methanol and 3 mL of water. The cartridge was then rinsed with 3 mL of water and dried for 10 min with vacuum. The analytes were eluted with 3 mL of 175 mM ammonium formate (pH 3). The eluate was then vortexed, filtered, and injected into the LC-MS/MS system.

3 Results and discussion

3.1 Method development

Aminoglycosides are basic and poorly soluble in organic solvents; however, they are highly soluble in aqueous solutions. In HILIC, acetonitrile is typically used as the weak injection solvent. However, in order to avoid a solvent change after the SPE cleanup, water, and mobile phase A (175 mM ammonium formate) were checked as injection solvents. Aqueous solutions with an injection volume of 5 μL gave good peak shapes and better sensitivity than using acetonitrile as the injection solvent. This could be because of the low solubility of aminoglycosides in acetonitrile.

Strong cation exchange SPE (Oasis MCX) was initially used to extract aminoglycosides from honey. Dissolving the weighed honey sample in 2% phosphoric acid and loading it onto Oasis MCX gave poor recoveries. This may be because aminoglycosides strongly bind to the strong cation exchange phase, as described in the literature [21, 22]. In the case of WCX SPE, four cartridges (Bakerbond WCX, Strata X CW, Accell Plus CM, and sampliq WCX) were examined. Twenty milliliters of water had to be used to dissolve the honey to prevent clogging the cartridge with the matrix. Formic acid (1, 2, and 5%) in water, 175 mM ammonium formate (pH 3 and 4.5) and formic acid in methanol (1%) were assessed as eluting solutions. Elution with formic acid in methanol was followed by evaporation and reconstitution with the aqueous mobile phase. A total of 175 mM ammonium formate (pH 3) eluted the analytes better than other solvents. Moreover,

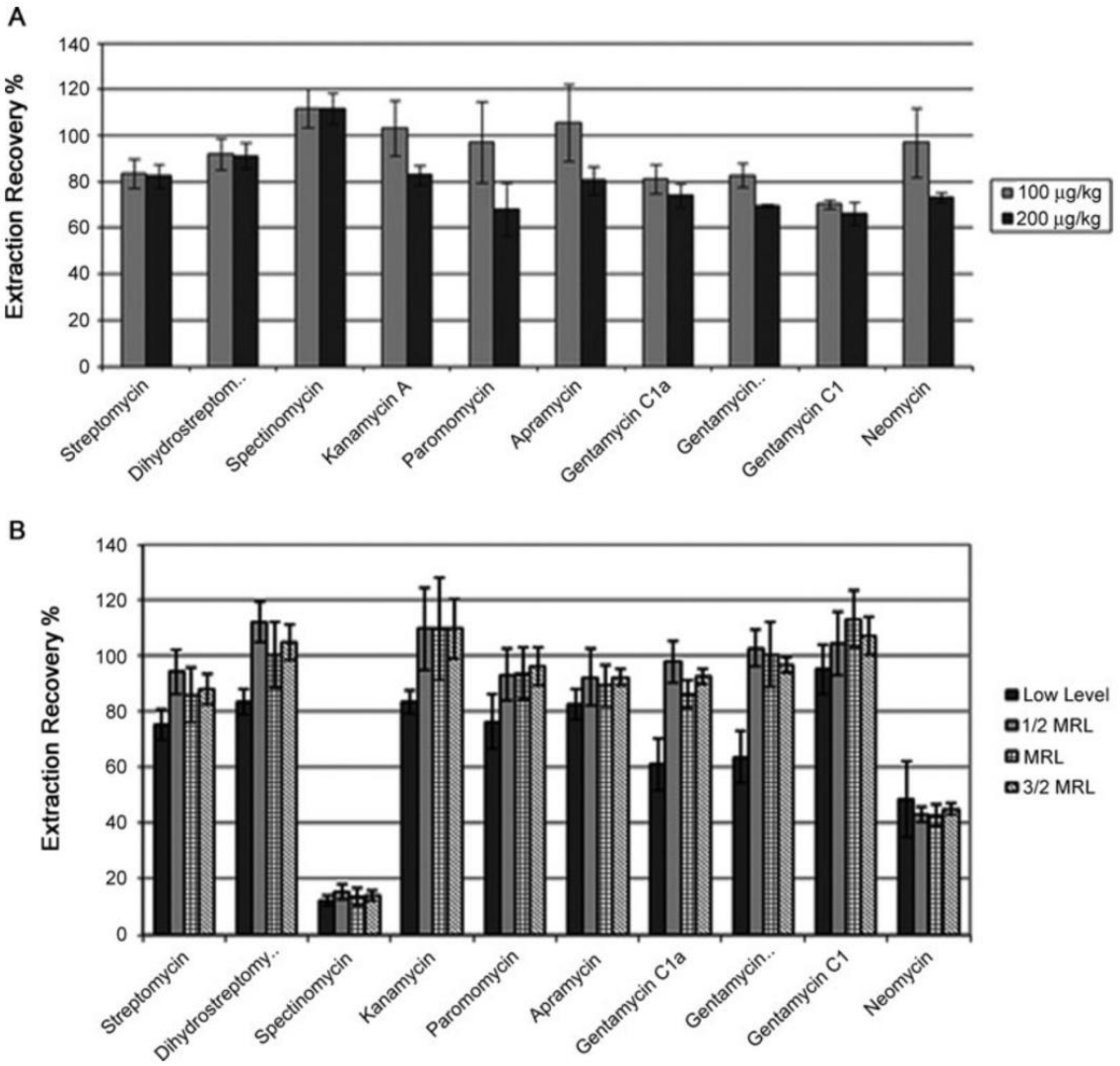

Figure 2. Extraction recoveries of aminoglycosides in honey matrix ($n = 3$ replicates) at two different concentrations (A). Extraction recoveries of aminoglycosides in kidney matrix ($n = 3$ replicates) at four different concentrations (B).

elution with the buffer made it possible to inject the eluate directly into the LC and therefore avoid the need for an evaporation step. Among the cartridges studied, Accell plus CM gave the best recoveries (Fig. 1). Extraction recoveries were estimated by spiking samples with analytes at 100 µg/kg and 200 µg/kg at the beginning of the extraction process and after the clean up. Extraction recoveries were calculated in triplicates by comparing peak areas and the results are shown in Fig. 2.

Extraction of aminoglycosides from tissue matrix generally requires acidic conditions, as they are tightly bound to proteins. An aqueous extraction solution of 10 mM ammonium acetate/0.4 mM EDTA/1% NaCl/2% TCA was adapted from the literature [www.fsis.usda.gov/PDF/CLG_AMG_2_03.pdf]. The concentration of TCA, a protein precipitating agent, is crucial, since a low concentration would not extract matrix-bound analytes properly, particularly neomycin. However, a high concentration would result in a high ionic strength and would therefore affect the retention of spectinomycin in the cartridge during SPE [8, 21]. The role of EDTA in the extracting solution is less clear. According to the literature [8, 21], it is added to counteract the ability of the analytes to bind with metal ions. However, the chelating capacity of EDTA at the low pH of the solution must be low. In any case, its presence is necessary from a practical point of view, since it obtains good recoveries. Extraction recoveries were calculated as mentioned above at the lowest levels of the

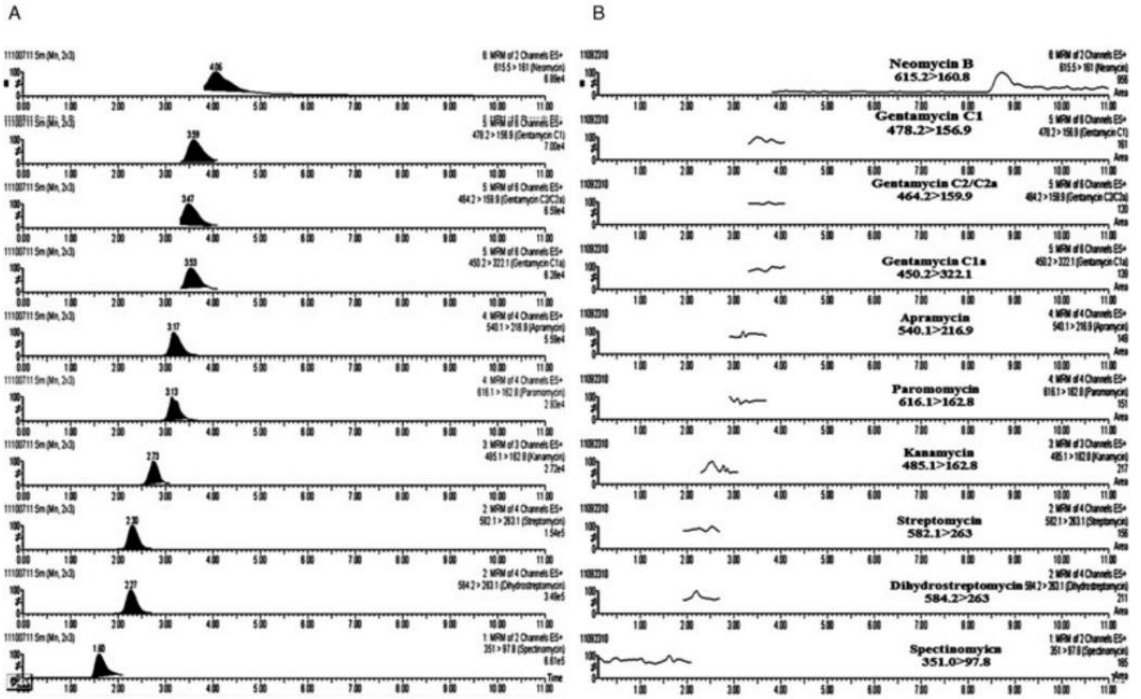

Figure 3. Chromatogram of a blank kidney sample spiked at MRL levels: spectinomycin at 5000 μg/kg, dihydrostreptomycin at 1000 μg/kg, streptomycin at 1000 μg/kg, kanamycin at 2500 μg/kg, paromomycin at 1500 μg/kg, apramycin at 20 000 μg/kg, gentamycin C1a, gentamycin C2/C2a and gentamycin C1a at 750 μg/kg, neomycin B at 5000 μg/kg (A). Chromatogram of a blank kidney sample (B).

calibration curves, 1/2 MRL, MRL, and 3/2 MRL levels (kidney), and the results are shown in Fig. 2. A low recovery of 14% for spectinomycin was observed. Since spectinomycin is a sensitive analyte and the recoveries were stable (precision at MRL level <10%), the extraction method was not changed.

Four HILIC stationary phases (bare silica, amino, amide, and zwitterionic phase), mobile-phase conditions (pH and ionic strength), and column temperature were assessed to achieve good separation, peak shapes, and sensitivity of aminoglycosides, which is described in detail elsewhere [29]. A total of 175 mM ammonium formate (pH 4.5) as the aqueous mobile phase and ZIC HILIC as the stationary phase were found to be optimal for the separation. Chromatographic separation of aminoglycosides was required to prevent crosstalk in the mass spectrometer. Chromatograms of spiked honey and kidney samples are shown in Supporting Information Fig. S2 and Fig. 3, respectively, together with the chromatograms of the corresponding blank samples.

3.2 Method validation

The methods were validated according to the Commission Decision 2002/657/EC [30] and its guidelines SANCO/2004/2726 [31]. The parameters evaluated were linearity, LOQ, trueness, precision, specificity, CCα, and CCβ. Tables 1 and 2 show the validation data of the methods for honey and kidney, respectively.

3.2.1 Linearity and LOQ

Quantification was carried out with calibration standards prepared by spiking blank samples before extraction with the analytes at different concentration levels and amikacin as the internal standard. Linearity was assessed over three days at five concentration levels. For all the analytes, R^2 was higher than 0.99 and the deviation of each point from the calibration line was lower than 15%, except for the lowest point. According to the validation procedure established in the Public Health Agency Laboratory of Barcelona, the lowest point in the calibration curve was set at a concentration that gave a deviation from calibration curve of about 25%. LOQ, defined as the concentration that gave a signal-to-noise ratio of 10, was estimated by injecting matrix-matched standards obtained by spiking blank matrix extracts at low concentrations.

3.2.2 Trueness and precision

Intraday precision was assessed at the four levels shown in Tables 1 (honey) and 2 (kidney) with six replicates. Interday precision was assessed at the same levels with six replicates

Table 1. Validation data for honey

Analyte	LOQ (μg/kg)	CC alpha (μg/kg)	CC beta (μg/kg)	Linearity range assessed (μg/kg)	Interday precision ($n = 3$ days)								Trueness [a] (error%)
					Level (μg/kg)	RSD%	Level (μg/kg)	RSD%	Level (μg/kg)	RSD%	Level (μg/kg)	RSD%	
Kanamycin A	41	50	67	70–495	70	17	150	19	300	13	450	13	14
Neomycin B	125	64	99	150–495	–	–	150	16	300	11	450	6	7
Gentamycin C1a	12	23	35	40–495	40	25	150	19	300	13	450	10	14
Gentamycin C2/C2a	24	28	42	40–495	40	26	150	15	300	8	450	10	10
Gentamycin C1	8	39	49	40–495	40	22	150	14	300	16	450	11	19
Paromomycin	23	45	66	70–495	70	23	150	16	300	19	450	10	20
Apramycin	12	22	36	70–495	70	13	150	14	300	8	450	14	9
Dihydrostreptomycin	2	13	17	20–495	20	16	150	16	300	9	450	13	9
Streptomycin	7	48	57	20–132	20	12	40	14	80	8	120	16	9
Spectinomycin	13	18	26	20–495	20	26	150	17	300	7	450	7	14

a) Average error% of four levels.

Table 2. Validation data for kidney

Analyte	LOQ (μg/kg)	CC alpha (μg/kg)	CC beta (μg/kg)	Linearity range assessed (μg/kg)	Interday precision ($n = 3$ days)								Trueness [b] (error%)
					Level (μg/kg)	RSD%	Level (μg/kg)	RSD%	Level [a] (μg/kg)	RSD%	Level (μg/kg)	RSD%	
Kanamycin A	85	2733	2965	200–4375	200	21	1250	8	2500	6	3750	4	2
Neomycin B	264	5164	5327	300–8750	300	9	2500	6	5000	2	7500	2	1
Gentamycin C1a	59	798	846	200–1312.5	200	15	375	9	750	4	1125	5	4
Gentamycin C2/C2a	70	807	864	200–1312.5	200	13	375	10	750	5	1125	6	2
Gentamycin C1	94	807	865	200–1312.5	200	12	375	10	750	5	1125	7	3
Paromomycin	117	1861	2223	200–2625	200	15	750	20	1500	16	2250	13	8
Apramycin	112	22 864	25 728	200–35 000	200	20	10 000	14	20 000	9	30 000	11	11
Dihydrostreptomycin	25	1178	1356	200–1750	200	20	500	14	1000	10	1500	9	11
Streptomycin	48	1153	1306	200–1750	200	12	500	12	1000	8	1500	13	7
Spectinomycin	151	5847	6695	200–8750	200	20	2500	6	5000	10	7500	7	8

a) Level corresponds to MRLs set by EC 37/2010.
b) Average error% of four levels.

per day on three different days. Trueness was calculated as the percentage of error between spiked and found concentrations (mean of 18 replicates at each level).

3.2.3 Specificity

Specificity was assessed by verifying the presence of interferences at the retention times of the analytes, above a signal-to-noise ratio of three. For this purpose, different kidney (bovine, ovine, and swine), liver, and blank fish samples, as well as honey samples from six different origins, were analyzed. Moreover, blank samples were spiked with the internal standard (amikacin) to assess the potential influence of different matrices on the internal standard signal. An RSD% lower than 5% was observed, showing that there are no significant differences between different matrices.

3.2.4 CCα and CCβ

When an MRL is set for an analyte/matrix combination, decision limit, CCα, is calculated using precision data obtained by analyzing 18 blank samples spiked at MRL, according to the equation CCα = MRL + 1.64 S (S = standard deviation at MRL level in terms of concentration). Detection capability, CCβ, is calculated from the CCα value according to the equation, CCβ = CCα + 1.64 S. As CCα is close to MRL, standard deviation at the MRL was used to estimate CCβ.

For banned substances, where an MRL has not been set, as is the case with honey, CCα is calculated from the calibration curve prepared by spiking blank matrices at four levels in the low concentration range. CCα is calculated as the concentration corresponding to the *y*-intercept plus 2.33 times its standard deviation. Subsequently, CCβ is calculated from the standard deviation obtained at the CCα level (CCα + 1.64 S).

4 Concluding remarks

Supporting Information Table S2 compiles recently published multiresidue methods for the analysis of aminoglycosides. It can be seen that IPC has been explored more extensively than HILIC for this purpose. Regarding recoveries, precision, and linearity ranges, no significant differences have been observed between the approaches. Trueness data are scarce but show a similar trend. Concerning LOQ, IPC methods show lower values than HILIC methods. However, the LOQ values reported in the present paper for kidney analysis are well below the MRLs established. Regarding the analysis of honey, the method reported here is the first to analyze multiple aminoglycosides based on HILIC and most of the LOQ values are lower than 25 μg/kg.

The methods proposed in the present paper are simpler than those previously reported. The use of an aqueous solution as the injection solvent makes it possible for the eluate of the SPE cartridge to be injected directly into the LC system without further treatment such as pH adjustment, evaporation, or reconstitution. Specifically, the method proposed by Zhu et al. [16] for the analysis of honey involves two SPE cleanup steps.

In conclusion, the proposed methods do not represent a dramatic improvement in relation to the existing methods, but they can be considered a valid alternative. The methods are currently used for routine analysis in the Public Health Agency Laboratory of Barcelona and have been included in the scope of accreditation (EN ISO/IEC 17025:2005). So far, one incurred sample has been found for neomycin B (1171 μg/kg) in kidney. Moreover, the laboratory participated in a proficiency test (FAPAS) for analyzing streptomycin and dihydrostreptomycin in honey at concentrations of about 40 μg/kg, with good results.

This study was supported by the Spanish Ministerio de Ciencia e Innovación (Project AGL 2008–05578-C05–03). P. Kumar thanks the Generalitat de Catalunya for an FI grant (Grant Number 2011 FI-B1 00141).

The authors have declared no conflict of interest.

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Supporting Information

Table S1. MRLs of aminoglycosides in bovine kidney.

Pharmacologically active substance	MRL (µg/kg)[a]	MRL (µg/kg)[b]
Kanamycin	2500	
Neomycin	5000	10000
Gentamycin	750	5000
Paromomycin	1500	
Apramycin	20000	
Dihydrostreptomycin	1000	1000[c]
Streptomycin	1000	
Spectinomycin	5000	5000

a MRLs set by EU 37/2010

b MRLs set by Codex alimentarius commission

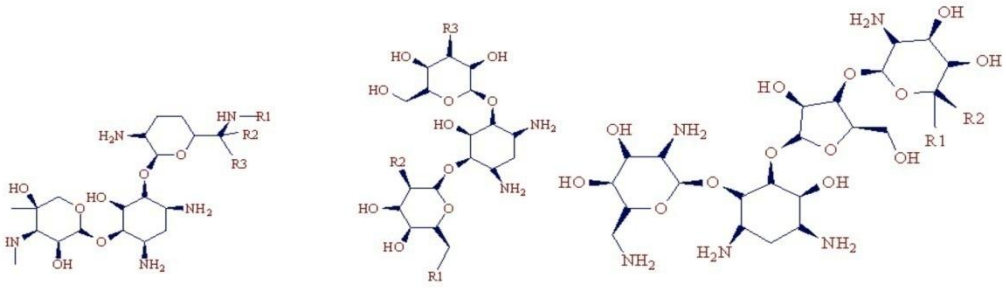
c Sum of streptomycin and dihydrostreptomycin

Table S2 . Review of multiresidue methods for aminoglycosides

Analytes	Matrix	Separation mode	Extraction recoveries	Interday precision (RSD %)	Trueness	LOQ	Linearity range	Reference
Spect, strep, DHS, amik, kana B, paro, apra, tobra, siso, neo B, genta C1	Veal liver	IPC	50 - 86%	5 - 19 %	-	-	100 - 25000 µg/kg	[24]
Spect, strep, DHS, amik, kana B, paro, apra, tobra, siso, neo B, genta C1, genta C1a, genta C2/C2a/C2b	Kidney	IPC	53 - 93%	2 - 16% (intraday)	-	1 - 60 µg/kg	100 - 5000 µg/kg	[23]
	Liver	IPC	48 - 87%	3 - 48%	-	1 - 30 µg/kg	100 - 5000 µg/kg	
Spec, DHS,strep, kana, genta C2/C2a, genta C1, apra, neo	Kidney	HILIC	72 - 101%	7 - 13%	0.7 - 12%	19 - 108 µg/kg	0-2µg/g	[10]
DHS, strep, genta C1, neo, tobra	Veal kidney	IPC	51 - 76%	12 - 19%		0.3 - 1.2 µg/kg	50 - 5000 µg/kg	[21]
Spect, strep, DHS, kana A, paro, apra, gent, neo B	Porcine kidney	IPC	48 - 80%	2.7 - 15 %	94 - 111%	-	0.25 MRL - 4 MRL	[20]
Strep, DHS, neo, paro, kana, amik, tobra, apec, apra, gent C1, gent C2, gent C1a	Kidney	IPC	68 - 100%	5 - 12%	-	-	0 MRL-10 MRL	[18]
	Honey	IPC	61 - 108%	9 - 16%	-	9 - 41 µg/kg (CCα)	0 CCα -10 CCα	
Strep, DHS, neo B, paro, kana, spec, apra, gent C1, gent C2/C2a, gent C1a	Honey	HILIC	68 - 112%	6 - 27%	7 - 20%	2 - 125 µg/kg	20 - 495 µg/kg	Current article
	Kidney	HILIC	14 - 105 %	2 - 21%	1 - 11%	25 - 264 µg/kg	200 - 35000 µg/kg	

Table S3. Multiple Reaction Monitoring (MRM) conditions of the method.

Analyte	Cone voltage (V)	Quantification trace (m/z)	Collision energy (eV)	Confirmation trace (m/z)	Collision energy (eV)
Spectinomycin	35	351.0>333.1	18	351.0>97.8	30
Streptomycin	55	582.1>263.0	30	582.1>221.2	40
Dihydrostreptomycin	55	584.2>263.0	30	584.2>246.0	40
Kanamycin A	25	485.1>162.8	30	485.1>324.1	15
Paromomycin	40	616.1>162.8	30	616.1>202.9	30
Apramycin	30	540.1>216.9	30	540.1>378.1	20
Neomycin B	50	615.2>160.8	30	615.2>163.0	35
Gentamycin C1a	25	450.2>322.1	15	450.2>159.9	20
Gentamycin C2/C2a	20	464.2>159.9	25	464.2>322.1	10
Gentamycin C1	30	478.2>156.9	20	478.2>322.1	15
Amikacin (internal standard)	30	586.1>425.0	20		



Genta	R1	R2	R3
C1	CH_3	H	CH_3
C1a	H	H	H
C2	H	H	CH_3
C2a	H	CH_3	H

Kana	R1	R2	R3
A	NH_2	OH	NH_2
B	NH_2	NH_2	NH_2
C	NH_2	OH	OH

Neo	R1	R2
B	CH_3-NH_2	H
C	H	CH_3-NH_2

Figure S1. Structures of Gentamycin C1, C1a and C2/C2a, Kanamycin A, B and C, Neomycin B and C.

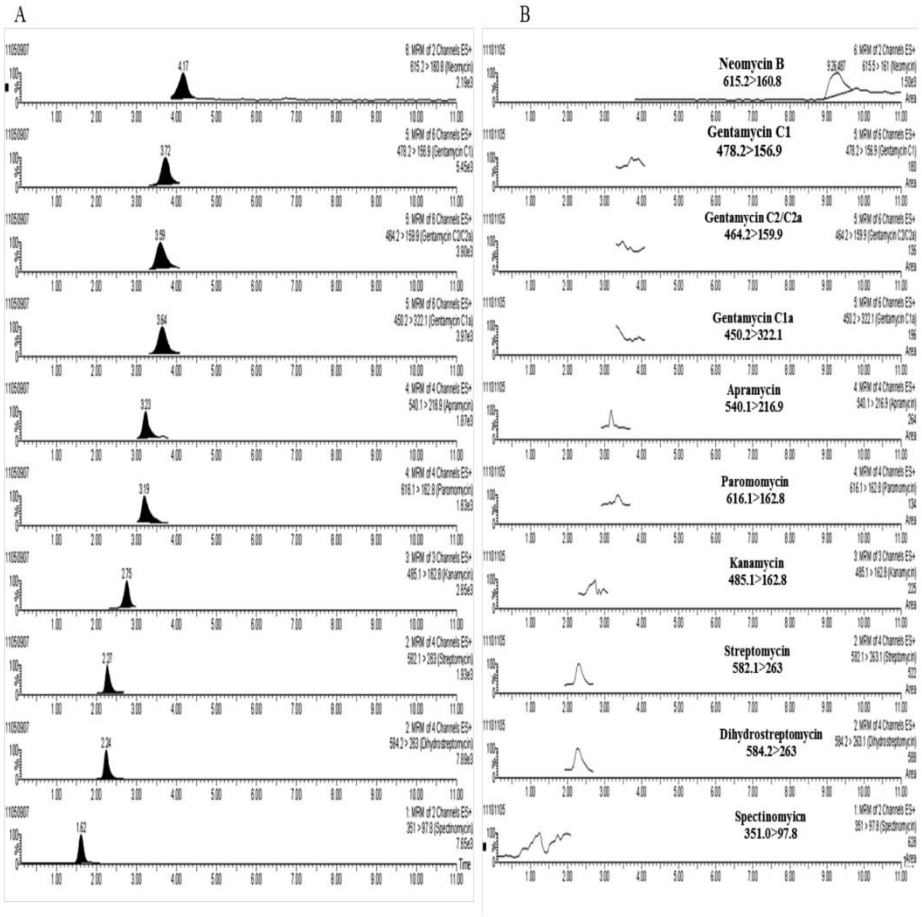


Figure S2. Chromatogram of a blank honey sample spiked at low levels: Spectinomycin, dihydrostreptomycin and streptomycin at 20 µg/kg, kanamycin, paromomycin and apramycin at 70 µg/kg, gentamycin C1a, gentamycin C2/C2a and gentamycin C1a at 40 µg/kg, Neomycin B at 150 µg/kg (Figure S2a). Chromatogram of blank honey sample (Figure S2b).

5.3. Discussion

For the sample cleanup step, four different SPE varieties of weak cation exchange (carboxylic acid functional group) were studied and the extraction recoveries for honey are reported in the article III. Waters Accella Plus CM gives better recoveries than the other cartridges studied. Strata XCW gives very low recoveries for gentamycins and neomycin. Looking at the difference between physical characteristics of SPE cartridges in table 5.3, the good recoveries with Accella Plus CM could be attributed to the silica support which provides silanol interactions to retain polar compounds. In polymeric support such silanol interactions will not be present. The low recoveries with strata XCW could be because of the low particle size (µm) which gets the honey matrix clogged easily.

Table 5.3. Physical characteristics of the SPE phases studied.

Commercial name	Chemistry	Support	Volume	Weight	Particle size (um)
Bakerbond (J.T.Baker)	WCX	Polymeric	3 ml	500 mg	47-61
Waters Accell Plus CM cartridges (Waters)	WCX	Silica	6 ml	500 mg	53.7
Strata X CW (Phenomenex)	WCX	Polymeric	6 ml	500 mg	33
SampliQ WCX (Agilent)	WCX	Polymeric	6 ml	150 mg	60

Concerning the chromatographic separation and the detection, in the case of aminoglycosides studied, the use of low resolution triple quadrupole mass spectrometer cause crosstalk between isobaric product ions when there is no enough separation of the analytes, which leads to error in quantitation. When two successive MRM transitions share a common daughter ion and the collision cell is not effectively cleared during the inter-channel delay, a false signal contribution will occur between the transitions. A sufficient interchannel delay time is required to allow ions to be cleared from the collision cell. The figure 5.4 (top) shows the expected behaviour of analytes and internal standard signals. Figure 5.4 (bottom) shows the peak area of internal standard, tobramycin with interchannel and interscan delay of 0.01/0.01 seconds has more crosstalk than with 0.05/0.02 seconds. Therefore amikacin was chosen as internal standard which does not share identical precursor or product ions with analytes. This phenomenon seems to be instrument specific and the new generation triple quadrupole analyzers report to overcome this crosstalk issue.

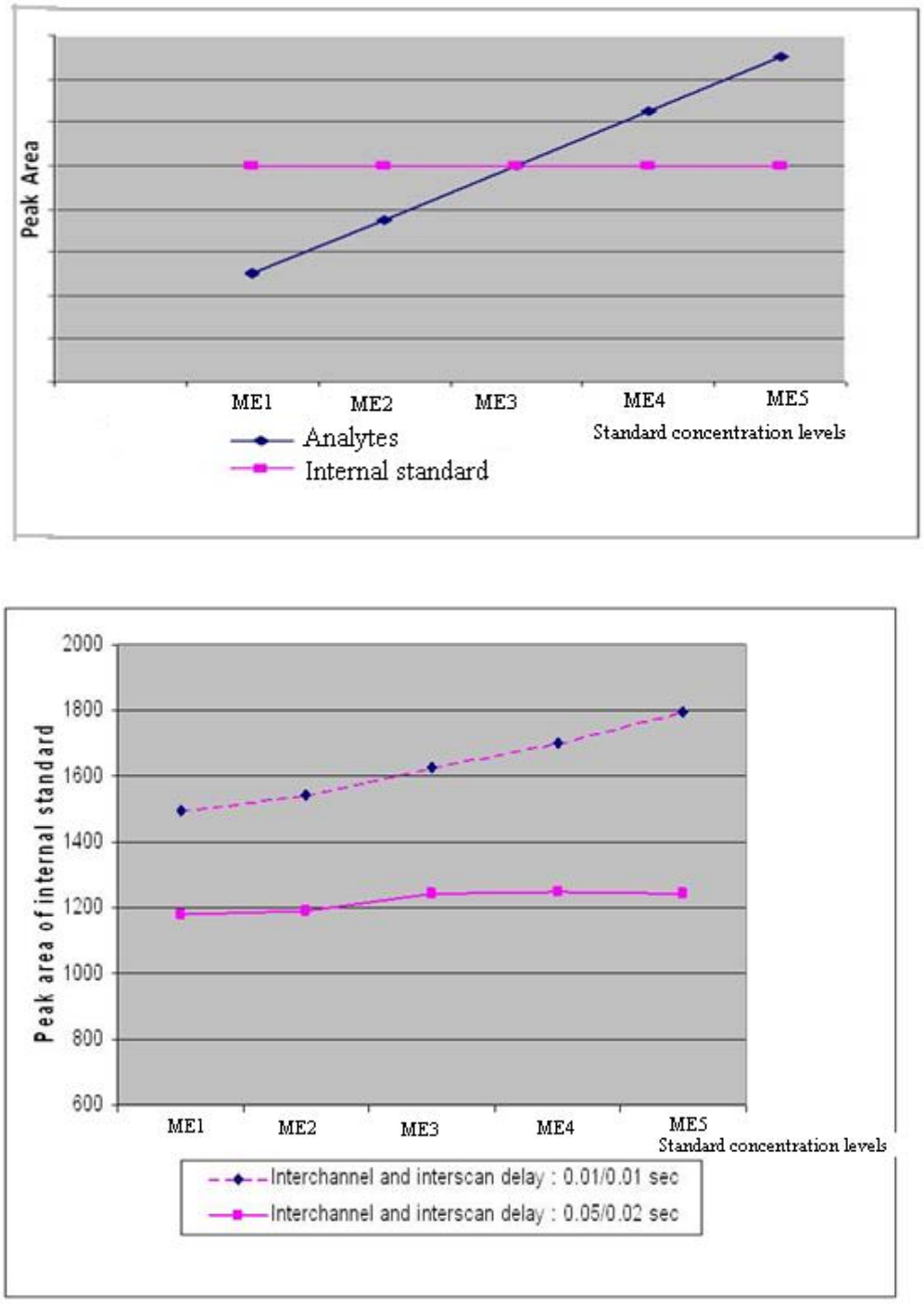


Figure 5.4. A typical calibration curve of an analyte and peak area of internal standard at various concentration levels (top) and peak area of tobramycin at various concentration levels of analyte at two interchannel and interscan delay settings (bottom).

The workflow of the method for analyzing kidney and honey are shown in figure 5.5 and figure 5.6 respectively. In both the methods, the evaporation step after SPE has been avoided, which gives high throughput of sample preparation and also would minimize the effects of matrix such as ion suppression as they are not concentrated by evaporation. It was possible to avoid evaporation step because, the SPE eluting solvent was chosen to be the same as the chromatographic mobile phase.

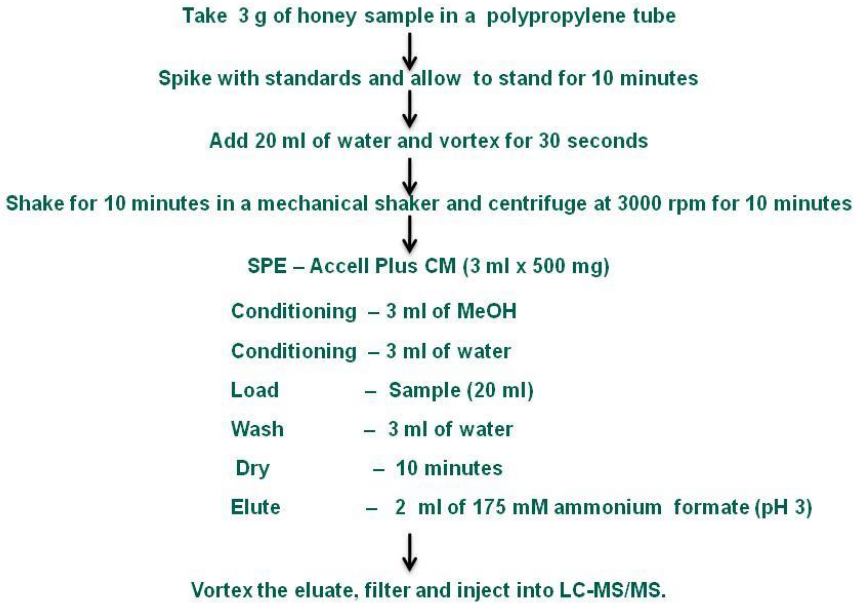


Figure 5.5. Workflow for analyzing aminoglycosides in honey

The performance characteristics of both the methods are summarized in table 5.4 and table 5.5. It could be noted that CCα and CCβ values of aminoglycosides in the method for honey are lower than the values in the method for kidney analysis. This is because the MRL values for aminoglycosides in kidney are high and in honey samples zero tolerance applies as there are no MRLs established.

Apart from the methods summarized in the articles, some more methods based on IPC [117, 118] and HILIC [119-121] have been published, reporting some improvements. Concerning the sample preparation, Ji et.al. [120] synthesized a molecularly imprinted polymer (MIP) sorbent using streptomycin as template to selectively extract aminoglycosides from the sample. MIP sorbents are generally more selective than other SPE sorbents. However, it has to be noted that MIP sorbents for all the aminoglycosides are not commercially available yet. In the chromatography side, the

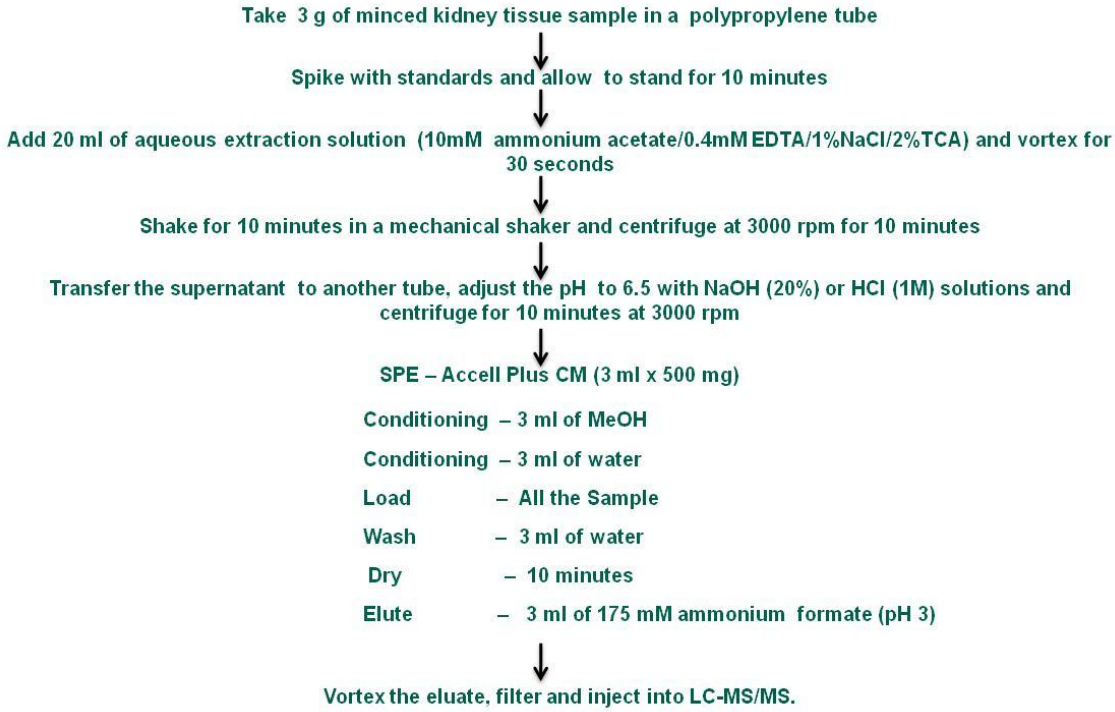


Figure 5.6. Workflow for analyzing aminoglycoside residues in kidney

Table. 5.4. Summary of performance characteristics of the method for honey analysis.

CCα	13-64 µg/kg
CCβ	17- 99 µg/kg
LOQ in sample	2 – 125 µg/kg
Trueness (as bias)	7-20%
Interday Precision at low concentrations (20-70 µg/kg)	12-26%

Table 5.5. Summary of performance characteristics of the method for kidney analysis

CC α	798-22864 µg/kg
CC β	846-25728 µg/kg
LOQ in sample	25-264 µg/kg
Trueness (as bias)	1-11 %
Interday Precision at low concentrations (200 & 300 µg/kg)	9-21 %

authors use mobile phases containing ion pairing agent while using C18 columns and high ionic strength (>100mM) mobile phases while using HILIC columns. However, Ji et al, use low ionic strength mobile phase (20mM ammonium formate) with amide HILIC column [120]. The method analyzes four aminoglycosides (streptomycin, dihydrostreptomycin, gentamycin and spectinomycin) and they coelute in chromatography. Lehotay et.al. [117] reports the structural elucidation of fragments for the first time during method development using QTOF, in order to avoid the chance of choosing wrong fragments and utilize triple quadrupole mass analyzer for quantitation and confirmation. Moreover, the article also reports that paromomycin transforms to neomycin due to chemical conversion which would affect the quantification of those analytes and therefore did not include paromomycin in validation studies. The authors followed the transitions, 615.3196 m/z > 161.0921 m/z, 615.3196 m/z > 163.1077 m/z (exact masses) for neomycin and 616.3036 m/z > 161.0921 m/z, 615.3036 m/z > 163.1077 m/z for paromomycin. The product masses are identical and normally these two analytes coelute in chromatography. It could be that crosstalk is the reason for the misidentification of these two analytes rather than the chemical conversion reported by the authors.

5.4. Analysis of aminoglycosides with amide HILIC-MS/HRMS

5.4.1. Introduction

Although the methods with HILIC reported earlier, avoid ion pairing agents and provide good results in the validation study, proficiency testing and routine analysis of samples, the method still requires a high ionic strength aqueous mobile phase (175 mM). This is generally not preferred to be used in mass spectrometer as it can cause ion suppression. Moreover, the peak shapes of some late eluting analytes like neomycin show severe tailing. Because of this some authors argue in support to the use of ion pairing agents rather than HILIC [122, 123]. The peak tailing of late eluting peaks gets worse with multiple injections of sample in ZIC HILIC column. Moreover, from our experience while presenting the previous work in conferences and journals, many laboratories were interested in a robust method for analysing aminoglycosides. There is a need for a chromatographic method with a simple mobile phase and avoiding the crosstalk problems that takes place with low resolution MS/MS. From our previous experience, amide HILIC, although does not separate all the analytes, it provides the possibility of using a simple mobile phase. Also, the use of Q-Orbitrap HRMS instrument provides the possibility of using precursor ions for quantitation avoiding crosstalk between identical product ions. In that direction, an LC-MS/HRMS method based on amide functional group HILIC and Q-Orbitrap hybrid mass spectrometer was developed.

5.4.2. Materials and methods

A Thermo Accela Ultra High Performance Liquid Chromatography (UHPLC) system coupled with a Maylab Switch column manager and a Thermo high resolution Q Exactive mass spectrometer (Thermo, Bremen, Germany) were used. The chromatographic system was coupled to the mass spectrometer with a Heated Electrospray Ionization Source II (HESI II). The molecular formula of the analytes was acquired from Chemspider and the monoisotopic mass of the compounds was calculated using Qualbrowser in Xcalibur. A resolving power of 70,000 FWHM was chosen for precursor scan and 35,000 FWHM was chosen for product ion scans. Targeted SIM /data dependent MS/MS (tSIM/ddMS/MS) mode was used to acquire data.

Individual standards at a concentration of 10 μg ml^{-1} were infused with a syringe at a flow rate of 5 μL min^{-1} through a T piece connected to an LC system with a mobile phase flow rate of 50 μL min^{-1}. Using the Q Exactive tune application, the precursor ion was selected in the quadrupole (tMS/MS mode) and product ions were found by increasing the Normalized Collision Energy (NCE). The actual higher energy collisional dissociation (HCD) energy in eV is calculated on the basis of the chosen NCE, mass and charge of the precursor ion [16]. After choosing the product ions, fragmentation energy scans were carried out to obtain the optimal NCE for complete fragmentation of precursor ions. The calculated monoisotopic mass of the product ions was obtained using the Mass Frontier software. An electron mass of 0.00054858 was taken into account to calculate monoisotopic masses. The monoisotopic masses and optimal NCEs of the monitored ions are shown in table 5.6. The optimized HESI II conditions were: spray voltage, 3.5 kV and 3.0 kV for positive and negative modes, respectively; sheath gas flow rate (N_2), 35 units; capillary temperature, 300 °C; S lens RF level, 50; heater temperature, 350 °C. Nitrogen obtained from a nitrogen generator – Zefiro (Clan Tecnologica, Seville, Spain) was employed as collision gas and damping gas.

The mass calibration of Orbitrap was performed every three days to ensure a working mass accuracy of lower than 5 ppm. Pierce LTQ Velos ESI Positive ion and Pierce LTQ Velos ESI Negative ion calibration solutions from Thermo Fisher Scientific (Rockford, IL, USA) were used to calibrate the mass spectrometer.

XCalibur 2.2 software from Thermo Fisher Scientific (MA, USA) was used to control the instrument and for data processing. Q Exactive 2.0 SP 2 (tune application) from Thermo Fisher Scientific (MA, USA) was used to control the mass spectrometer.

Massfrontier 7.0 from HighChem, Ltd (Bratislava, Slovakia) was used to obtain the fragmentation pattern and exact mass of the product ions in positive mode.

Mobile phase A: 1% formic acid in water, Mobile phase B: water. The gradient (t mins, %A) used is as follows (0,45%) (3, 85%) (6,85%) (6.1, 45)(10, 45) was used. The LC column used was a TSK gel Amide 80 – 2 x150mm, 3μm (Tosoh, Tokyo, Japan).

Table 5.6. Precursor ion masses (Exact mass) and product ion masses (accurate mass) of the aminoglycosides studied.

Analyte	Precursor ion Monoisotopic exact mass (m/z)	Product ion Accurate mass (m/z)
Spectinomycin	333.1656	98.0602
Streptomycin	582.2729	263.1459
Dihydrostreptomycin	584.2886	263.1459
Kanamycin	485.2453	163.1076
Paromomycin	616.3036	163.1076
Apramycin	540.2875	217.1181
Gentamycin C1a	450.2922	160.0967
Gentamycin C2/C2a	464.3079	160.0967
Gentamycin C1	478.3235	157.1335
Neomycin	615.3196	161.0920
Amikacin	586.2930	

5.4.3. Results and Discussion

From the previous method development [124] it was known that using 1% formic acid in water as mobile phase effectively controls the silanol interactions in amide HILIC phase and achieve good peak shapes. The chromatogram of the analytes studied are shown in figure 5.7, where spectinomycin elutes first and rest of the analytes coelute together.

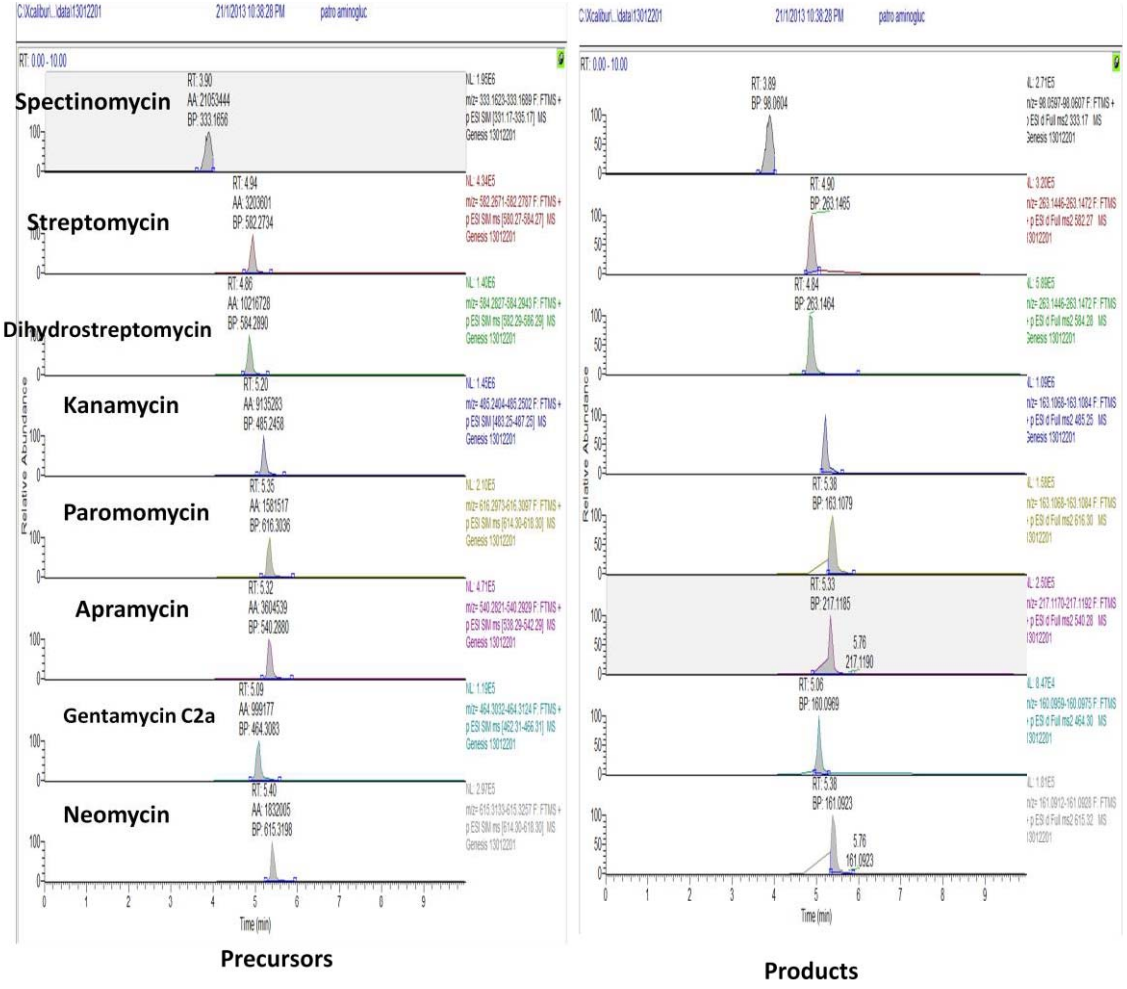


Figure 5.7. Chromatogram of aminoglycosides from an injection of 100 µg/l standard solution in the LC-MS/HRMS.

In the method with ZIC HILIC column, the late eluting peak neomycin has strong peak tailing (figure 5.8) after multiple injections of samples (approx. 100 injections). A chromatographic peak of neomycin at 300 µg/kg spiked kidney sample analyzed with amide HILIC stationary phase and quadrupole Orbitrap mass spectrometer is shown in the figure 5.9 with good peak shapes. Moreover, the method avoids the crosstalk between identical product ions as precursor ions are chosen as quantifier ion.

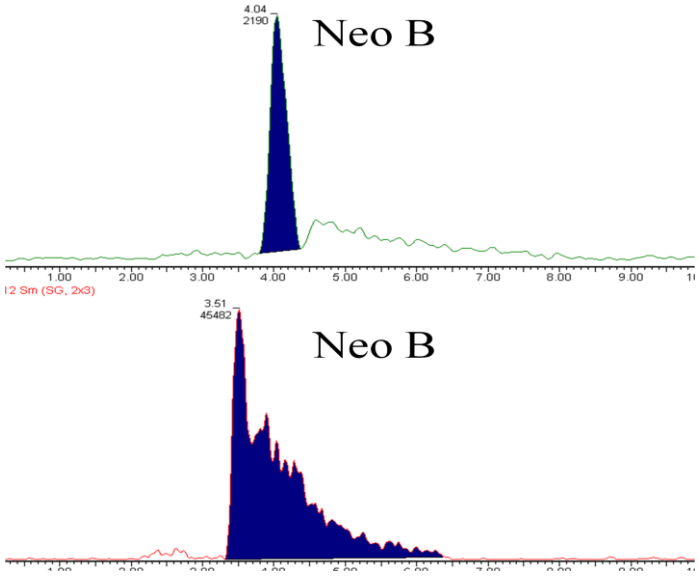


Figure 5.8. Chromatograms of neomycin B spiked kidney sample at 300 µg/kg with ZIC HILIC column coupled to triple quadrupole mass spectrometer. Chromatogram of neomycin B in a new column (top) and after multiple sample injections (below)

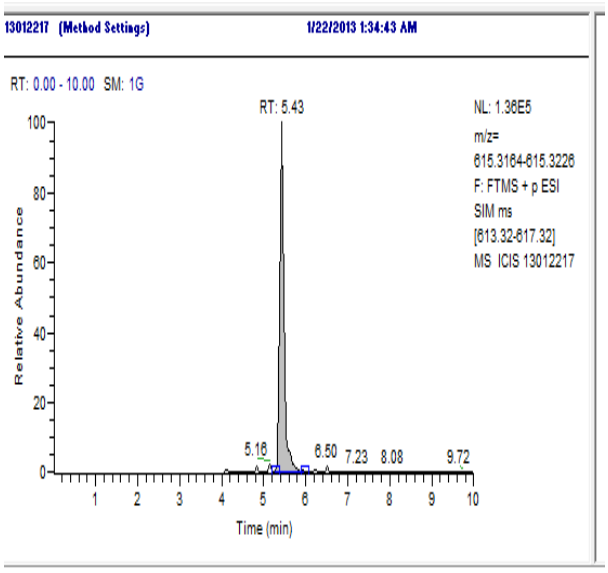


Figure 5.9. Chromatographic peak of neomycin B spiked kidney sample at 300 µg/kg with amide HILIC coupled to quadrupole Orbitrap hybrid mass spectrometer.

A positive sample for dihydrostreptomycin in kidney at a concentration above the MRL and outside the working concentration range was reinjected after identification with LC- QqQ method with this LC-MS/HRMS method and the chromatogram is shown in the figure 5.10. This shows the applicability of the method to real samples.

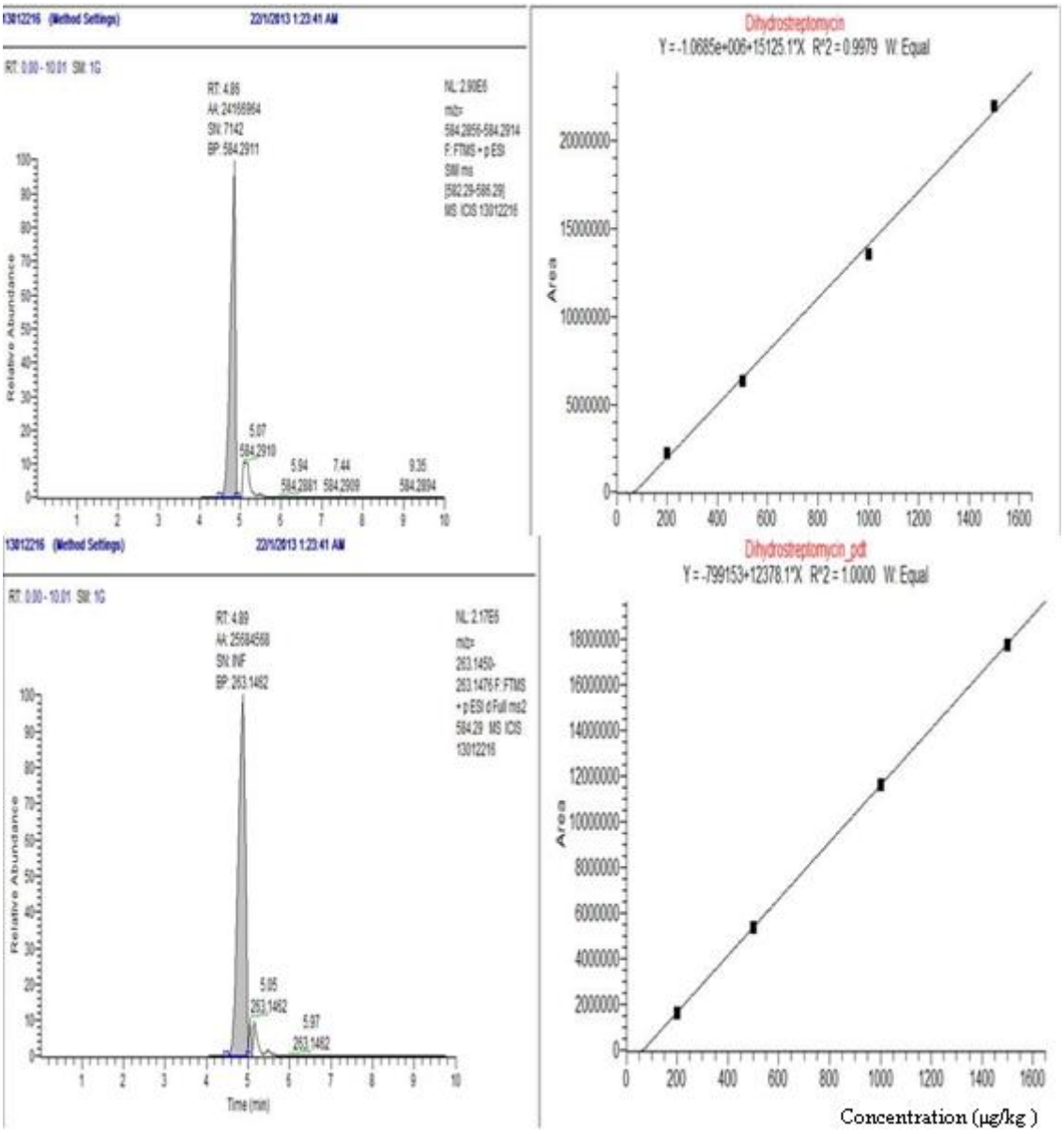


Figure 5.10. A non compliant kidney sample analyzed with amide HILIC – MS/HRMS with dihydrostreptomycin residue. Dihydrostreptomycin precursor ion and calibration curve (top), product ion and calibration curve (bottom).

Although the method is not validated yet, reinjection of samples from QqQ method showed sufficient sensitivity and linearity over the whole concentration range for all the analytes. These preliminary results are very promising, however applicability of this method for routine analysis will need a complete validation of the method.

6. Analysis of synthetic hormones in animal urine using LC-MS/HRMS

6.1. Introduction

The quadrupole Orbitrap (Q Exactive) instrument is a high resolution hybrid instrument. It was first introduced in 2011 and it was applied generally for research purposes in the fields of metabolomics, proteomics, food safety and environmental analysis. However, its use in official control laboratories for routine analysis is scarce. Triple quadrupole mass analyzers are still the workhorses in the field of testing laboratories because of their cost, ease to use and robustness. Previously HRMS instruments were less robust (frequent calibrations and high maintenance required) and often did not provide enough sensitivity to satisfy the needs of food testing laboratories. (Figure 6.1.) This new generation of Orbitrap instruments has increased ion current transmission efficiency, leading to high sensitivity. Moreover, the presence of a quadrupole to filter unwanted matrix interferences and isolate precursor ions, and the multiplexing option to increase the scan speed, provide interesting performance characteristics for food testing compared to single stage Orbitrap instrument (Exactive) [125]. Combining high resolving power, mass accuracy, sensitivity and full scan capability, it opens up new possibilities in food safety testing.

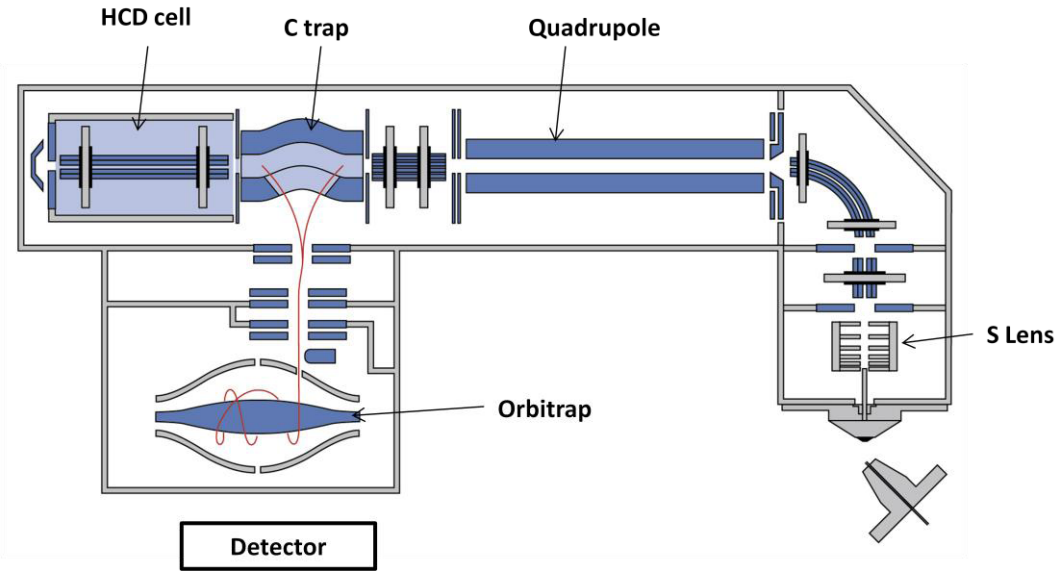


Figure 6.1. Schematic of quadrupole – Orbitrap hybrid instrument.

The presence of quadrupole offer different modes of acquisition especially data dependent acquisitions (DDA). Various instrument parameters (table 6.1) of the mass spectrometer have to be optimized to achieve optimal results. In that sense, a systematic study with some model analytes and difficult matrix combinations to find out optimal parameters for routine analysis deemed to be necessary. In that sense, an analytical methodology based on triple quadrupole analyzer to analyze hormones in urine, previously developed at the laboratory of ASPB was decided to be transferred to HRMS.

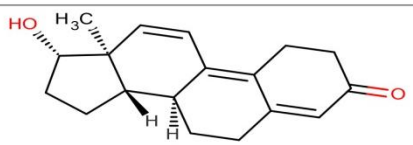
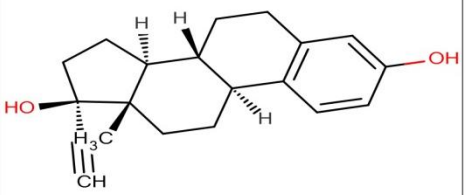
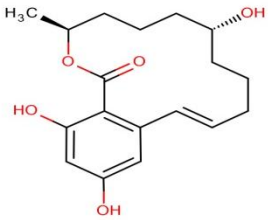
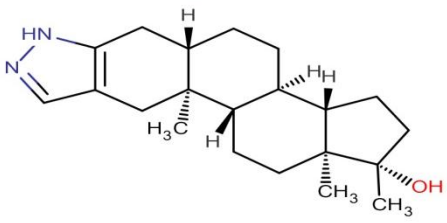
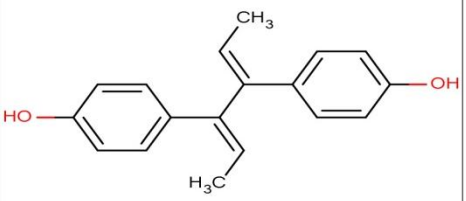
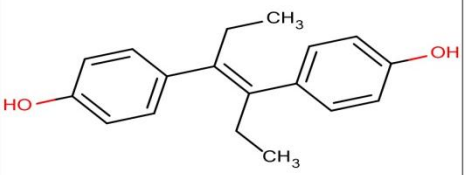
Table 6.1. Instrument parameters in the quadrupole Orbitrap instrument adapted from Randall et al [126].

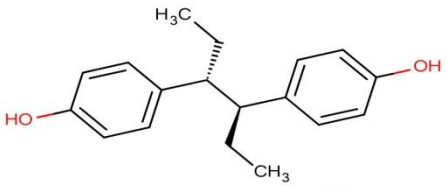
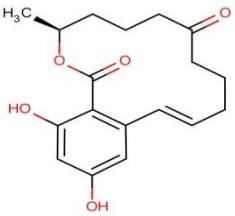
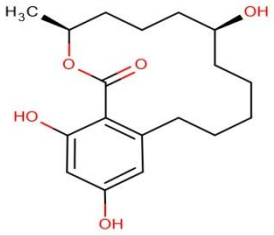
Parameter	Description
Apex trigger	Instrument triggers MS/MS at peak apex
Dynamic exclusion	Time m/z precursor ions are placed on exclusion list after initial sequencing
Isolation window	m/z window used for mass filtering prior to MS/MS scans
Lock mass	Internal real time calibration
AGC MS/MS	Target amount of charged ions to accumulate for MS/MS scans
MS/MS Events	Maximum number of data-dependent scans following precursor scan
IT MS/MS	Maximum allowed injection time to gather ions in C-trap
Normalized collision energy	Relative amount of energy required to fragment ions
Stepped NCE	Dynamic fragmentation energy
S-Lens	Voltage applied at inlet source
Underfill ratio	Threshold for MS/MS activation

Anabolic agents such as hormones and other hormonally active substances were used for increasing the growth rate in farm animals since 1950s. Due to the possible risk of cancer from the residues of these substances in many countries around the world their use is controlled or prohibited [18]. They are administered as feed additives, injection or rubber implants for slow release of the substance. However, anabolic agents are metabolized rapidly and an optimal concentration in blood circulation is necessary for a high growth rate. So, it is preferred to add them as feed additives. These substances are

rapidly excreted and hence, high concentrations can be found in bile, urine and faeces. So, urine becomes an ideal sample for testing and control [127]. The chemical structure and log K_{ow} of the hormones studied are summarized in table 6.2.

Table 6.2. List of synthetic hormones, their chemical structure, molecular formula, log K_{ow}, CAS no. and Chemspider ID.

Analyte	Structure	Molecular Formula	log Kow	CAS No.	Chemspider id
Trenbolone		$C_{18}H_{22}O_2$	2,65	10161-33-8	23383
17α Ethinylestradiol		$C_{20}H_{24}O_2$	4,12	57-63-6	5770
Zeranol		$C_{18}H_{24}O_5$	4,09	26538-44-3	4447689
Stanazolol		$C_{21}H_{32}N_2O$	4,42	10418-03-8	23582
Dienestrol		C18H18O2	5,43	84-17-3	580857
Diethylstilbestrol		$C_{18}H_{20}O_2$	5,64	56-53-1	395306

Hexestrol		$C_{18}H_{22}O_2$	5,60	84-16-2	166848
Zearalenone		$C_{18}H_{22}O_5$	3,58	17924-92-4	4444897
Taleranol		$C_{18}H_{26}O_5$	5,37	42422-68-4	58897

In the EU, the use of hormonally active substances as growth promoting agent in food producing animals is prohibited and hence MRLs are not established. Also, Codex has not set MRLs for these substances [128]. The acceptable daily intake values set by JECFA and the EU MRPL values are summarized in table 6.3 [129]. The community reference laboratory for stilbenes, steroids and resorcyclic acid lactones is National Institute for Public Health and Environment (RIVM), in The Netherlands.

Table 6.3. Synthetic hormones studied, their ADI and MRPLs set by CRL –RIVM, The Netherlands.

Classification by 96/23/EC	Analyte	ADI µg/kg	MRPL in urine by CRL, EU µg/kg
A1 Stilbenes	Diethylstilbestrol	not set	1
	Dienestrol	not set	2
	Hexestrol	not set	2
A3 Steroids	17α – Ethinylestradiol	not set	1
	Trenbolone	0-0.02	2
	Stanazolol	not set	2

A4 Resorcyclic acid lactones	Zeranol	0-0.5	2
	Taleranol	not set	2
	Zearalenone	not set	2

In an analytical perspective, urine is a difficult matrix with a lot of potential interferences and hence in this work, the capacities of the HRMS equipment are thoroughly explored and are reported in the following scientific article. The main objective of this work is to evaluate the performance of Q Exactive instrument for targeted analysis and the transfer of a QqQ method to HRMS for the analysis of synthetic hormones in urine at the laboratory of ASPB, Barcelona.

6.2. ARTICLE IV

Analytica Chimica Acta 780 (2013) 65–73

Contents lists available at SciVerse ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Targeted analysis with benchtop quadrupole–orbitrap hybrid mass spectrometer: Application to determination of synthetic hormones in animal urine

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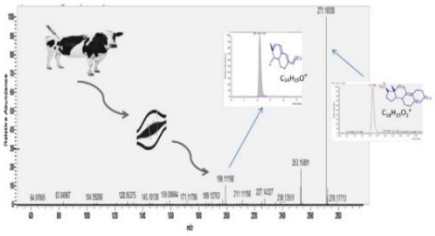
HIGHLIGHTS

- The quadrupole in Q Exactive acts as a powerful filter to reduce ion suppression.
- Reducing mass range using quadrupole in targeted modes increases the S/N ratio.
- Targeted SIM data dependent scan modes are the most suitable for residue analysis.
- A HRMS confirmatory method for synthetic hormones in urine has been developed.
- The Q Exactive provides similar sensitivity and enhanced selectivity compared to QqQ.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 December 2012
Received in revised form 5 April 2013
Accepted 10 April 2013
Available online 17 April 2013

Keywords:
Q Exactive
Synthetic hormones
Animal urine
Food safety control

ABSTRACT

Sensitive and unequivocal determination of analytes/contaminants in complex matrices is a challenge in the field of food safety control. In this study, various acquisition modes (Full MS/AIF, Full MS + tMS/MS, Full MS/dd MS/MS and tSIM/ddMS/MS) and parameters of a quadrupole–orbitrap hybrid mass spectrometer (Q Exactive) were studied in detail. One of the main conclusions has been that, reducing the scan range for Full MS (using the quadrupole) and targeted modes give higher signal-to-noise (S/N) ratios and thereby better detection limits for analytes in matrix. The use of Q Exactive in a complex case, for the confirmatory analysis of hormones in animal urine is presented. A targeted SIM data dependent MS/MS (tSIM/ddMS/MS) acquisition method for determination of eight synthetic hormones (trenbolone, 17α ethinylestradiol, zeranol, stanozolol, dienestrol, diethylstilbestrol, hexestrol, taleranol) and a naturally occurring hormone (zearalenone) in animal urine were optimized to have sensitive precursors from targeted SIM mode and trigger MS/MS scans over the entire chromatograph peak. The method was validated according to EC/657/2002. CCα (decision limit) for the analytes ranged between 0.11 $\mu g\,L^{-1}$ and 0.69 $\mu g\,L^{-1}$ and CCβ (detection capability) ranged between 0.29 $\mu g\,L^{-1}$ and 0.90 $\mu g\,L^{-1}$.

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http://dx.doi.org/10.1016/j.aca.2013.04.017

1. Introduction

In farming practices, the use of hormones and some drugs are prohibited, while specific limits have been established for other drugs [1]. These compounds must be monitored in food of animal origin and in the tissues and fluids of food-producing species. Currently, triple quadrupole mass analyzers (QqQ) have been the workhorse in control laboratories due to their sensitivity, selectivity and robustness. Although criteria for confirmation of analytes according to the decision 2002/657/EC [2] are generally met using triple quadrupole mass analyzer, the instrument is susceptible to false positive [3] and false negative [4] results due to its low resolution. Moreover, in the last decade advances in high resolution mass spectrometry (HRMS) instrumentation, such as TOF and Orbitrap, and the increasing requirements (resolving power, throughput, full scan analysis) of regulatory laboratories have led to a gradual increase in the use of HRMS instruments in routine analysis.

In general, requirements of a food safety laboratory from an HRMS equipment is to perform sensitive and selective analysis at low concentrations (usually <1 $\mu g\,kg^{-1}$) in the case of prohibited chemical substances, or full scan analyses to look for both multi-residue targeted (>100 analytes) and non-targeted analysis at much higher concentrations (usually >1 $\mu g\,kg^{-1}$).

Since the introduction of the first orbitrap-based instrument in 2005, a new line of orbitrap based HRMS instruments have been produced and are mainly being applied in the field of proteomics and also in metabolomics, drug discovery, drug, food and environmental testing. This is evident from the number of publications returned by Web of Science for the keyword "orbitrap", indicating a surge in the number of publications since 2005. The quadrupole–orbitrap (Q Exactive) hybrid instrument was first introduced in 2011 and only a few articles have been reported, and these are limited to the fields of proteomics [5–8] and drug testing [9]. Combining the mass selection/isolation capability of the quadrupole and the high resolution of orbitrap, this instrument has a great potential to avoid false compliant and non-compliant results in food safety testing.

The current legislative framework for residue analysis [2] in the EU is not adequate for the use of HRMS in analytical methodology, as it lacks criteria for accurate mass measurements. A few cases of false positive [3] and false negative results [4,10,11] have been reported in the literature, and are attributed to limitations in the confirmation criteria of Decision 2002/657/EC and also to the lack of resolution of triple quadrupole analyzers. This highlights the need to update the legislation, and also the need for the use of HRMS in food safety. Some studies have been carried out to establish criteria for HRMS analysis [12,13] and proposals have been made for updating the regulation [11,14]. Also, within Codex Alimentarius Commission (CAC), the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) is drafting a set of guidelines on "performance characteristics for multi-residue methods" [15], which include criteria for HRMS-based analytical methods. According to these draft guidelines, when HRMS instrument is used for confirmatory methods, the monitored ions should have a mass measurement accuracy lower than 5 ppm and a resolution higher than 20,000 full width at half maximum (FWHM) resolution at the mass range of interest. Each HRMS precursor ion earns two identification points and each HRMS transition product earns 2.5 identification points. At least one ion ratio must be monitored in order to eliminate the possibility of fragments arising from isobaric compounds or compounds with similar structure. Also, retention times or relative retention times should be monitored. For substances with maximum residue limits (MRL), a minimum of 3 identification points are required. For substances without MRL (banned substances), the number of identification points required to confirm them has not been established. Conversely, Decision 2002/657/EC requires a minimum of 4 identification points to confirm the banned substances. Tolerances for relative ion intensities in the CAC guidelines are shown in Table S1, Supplementary Data, where the relative tolerance for ion ratio is 20–50%, depending on the relative ion intensity of the peak. The tolerances for ion ratio criteria are debated as it can give rise to false negative cases, such as in the analysis of quinolone antimicrobials [10] and pesticides [11]. Mol et al. [11] stated that no relation between variability of the ion ratio and relative ion intensity was observed but variability increases with the decreasing concentration and moreover suggesting a wider tolerance for ion ratio criteria to avoid false negative cases.

The present work was carried out in the context of the implementation of a Q Exactive mass spectrometer in the routine work of the laboratory of Public Health Agency of Barcelona, an ISO 17025 accredited laboratory with a heavy workload. To date, HRMS has been mainly used for research purposes, but has not been widely applied to routine analysis. In this scenario, a detailed exploration of the capabilities of the Q Exactive instrument was necessary in order to establish protocols for the optimal use of the instrument in relation to the QqQ already working in the laboratory. With this purpose, this article discusses the various acquisition modes of Q Exactive for targeted analysis and the LC–MS/HRMS method development for analyzing eight synthetic hormones and a naturally occurring hormone in a complex urine matrix. A data dependent MS/MS acquisition method at high resolution was used in order to avoid post interface suppression due to the complex matrix. This hormones method was chosen to be migrated from MS to HRMS in order to have unequivocal confirmation of analytes, considering the complexity of the urine matrix.

2. Experimental

2.1. Chemicals and reagents

Standards of stanozolol, taleranol (β-zearalanol), zearalenone, zeranol (α-zearalanol), hexestrol, dienesterol, 17α-ethinylestradiol, diethylstilbestrol and trenbolone were obtained from Sigma–Aldrich (St. Louis, MO, USA). Commercial internal standard solutions (100 $mg\,L^{-1}$) of Nandrolone D3 and taleranol D4 were obtained from RIVM (The Netherlands). β-Glucuronidase EC 3.2.1.31 type HP-2, Sigma–Aldrich (St. Louis, MO, USA) and acetonitrile – hypergrade for LC–MS from Merck KGaA (Darmstadt, Germany) were used. Double deionized water (Milli Q, Millipore, Molsheim, France) of 18.2 $M\Omega\,cm^{-1}$ was used. All other reagents were of analytical grade.

Stock standard solutions of each target analyte at a concentration of 1000 $mg\,L^{-1}$ were prepared in acetonitrile: water (3:7) and stored up to 1 year at −20 °C. Internal standard was diluted using a mixture of acetonitrile: water (3:7) to 2 and 0.2 $mg\,L^{-1}$. The working solutions of standards at a concentration of 0.2 and 0.05 $mg\,L^{-1}$ were prepared with acetonitrile: water (3:7). The working solutions were stored up to 6 months at 8 °C. Oasis HLB cartridges (60 mg) in well plate format (12 × 8) (Waters Corp., Milford, MA, USA) were used. Samples were filtered with 0.2 μm Whatman Mini-UniPrep PVDF filters with polypropylene housing from GE Healthcare UK Ltd. (Buckinghamshire, UK).

2.2. Instrumentation

A Thermo Accela Ultra High Performance Liquid Chromatography (UHPLC) system coupled with a Maylab Switch column manager and a Thermo high resolution Q Exactive mass spectrometer (Thermo, Bremen, Germany) were used. The chromatographic system was coupled to the mass spectrometer with a Heated

Electrospray Ionization Source II (HESI II). The molecular formula of the analytes was acquired from Chemspider and the monoisotopic mass of the compounds was calculated using Qualbrowser in Xcalibur.

Individual standards at a concentration of 10 μg mL^{-1} were infused with a syringe at a flow rate of 5 μL min^{-1} through a T piece connected to an LC system with a mobile phase flow rate of 50 μL min^{-1}. Using the Q Exactive tune application, the precursor ion was selected in the quadrupole (tMS/MS mode) and product ions were found by increasing the Normalized Collision Energy (NCE). The actual higher energy collisional dissociation (HCD) energy in eV is calculated on the basis of the chosen NCE, mass and charge of the precursor ion [16]. After choosing the product ions, fragmentation energy scans were carried out to obtain the optimal NCE for complete fragmentation of precursor ions. The calculated monoisotopic mass of the product ions was obtained using the Mass Frontier software. An electron mass of 0.00054858 [17] was taken into account to calculate monoisotopic mass [18]. The monoisotopic mass and optimal NCEs of the monitored ions are shown in Table 1. The optimized HESI II conditions were: spray voltage, 3.5 kV and 3.0 kV for positive and negative modes, respectively; sheath gas flow rate (N_2), 35 units; capillary temperature, 300 °C; S lens RF level, 50; heater temperature, 350 °C. Nitrogen obtained from a nitrogen generator – Zefiro (Clan Tecnologica, Seville, Spain) was employed as collision gas and damping gas.

The mass calibration of orbitrap was performed every three days to ensure a working mass accuracy of lower than 5 ppm. Pierce LTQ Velos ESI Positive ion and Pierce LTQ Velos ESI Negative ion calibration solutions from Thermo Fisher Scientific (Rockford, IL, USA) were used to calibrate the mass spectrometer. A Hettich refrigerated centrifuge (Tuttlingen, Germany) was used.

XCalibur 2.2 software from Thermo Fisher Scientific (MA, USA) was used to control the instrument and for data processing. Q Exactive 2.0 SP 2 (tune application) from Thermo Fisher Scientific (MA, USA) was used to control the mass spectrometer.

Massfrontier 7.0 from HighChem, Ltd (Bratislava, Slovakia) was used to obtain the fragmentation pattern and exact mass of the product ions in positive mode.

2.3. Procedures

2.3.1. Study of MS parameters and flow rate

Three hormones (stanazolol, diethylstilbestrol and zeranol) were chosen as model analytes to study the MS parameters, one in positive (stanazolol) and two in negative mode (diethylstilbestrol and zeranol). Systematic experiments (Table S2, Supplementary Data) were carried out with a mixture of standards at 0.1 mg L^{-1} concentration and an injection volume of 2 μL. An Acquity UPLC® BEH C18 2.1 mm × 100 mm (1.7 μm) column (Waters, MA, USA) was used. The mobile phase was a mixture of water (A) and acetonitrile (B) at a flow rate of 300 μL min^{-1}. The elution gradient was (t, %B): (0, 10), (7, 75), (8, 75), (8.1, 100), (11, 100), (11.1, 10), (15, 10). Flow rate experiments were carried out with Acquity UPLC® BEH C18 1.0 mm × 100 mm (1.7 μm) and 2.1 mm × 100 mm (1.7 μm) columns (Waters, MA, USA) using the same gradient.

2.3.2. Analysis of hormones in urine

The sample preparation procedure for extracting hormones from urine was adapted from Rubies et al. [19] and is described as follows. 1.5 mL of urine was centrifuged at 10 °C, 10 min, 3000 rpm and the supernatant was pipetted into a 20 mL glass tube. Method matrix fortified calibration curve standards were prepared by adding the analytes to blank samples at a concentration range of 1–20 μg L^{-1}. Internal standards were added to samples and standards to give a final concentration of 6.7 μg L^{-1} urine. A 0.5 mL volume of acetate buffer (2 M, pH 4.8) and 25 μL β-glucuronidase (EC 3.2.1.31 type HP-2) were added to each tube and vortexed. Tubes were then placed in a heated oven at 55 °C for 60 min. After hydrolysis, samples and standards were left at room temperature, and SPE was performed. Oasis HLB cartridges in well plate format were conditioned with 1 mL methyl tert butylether (MTBE), 1 mL methanol, and 1 mL deionized water. After these steps, samples and standards were loaded onto cartridges. Cleaning steps were performed with 1 mL of 1:1 mixture of methanol: 2% formic acid in water, 1 mL deionized water, 1 mL of 1:9 methanol: 2% ammonia solution in water, followed by cartridge drying under vacuum for 5 min. Elution was performed using 1.5 mL solution of 1:9 methanol: MTBE. Eluates were collected in glass tubes and concentrated under nitrogen flow for 5 min at 45 °C to reduce volume to ~1 mL; then 1 mL deionized water and 2 mL of 1:1 mixture of diethyl ether: petroleum ether solution were added to each tube.

Tubes were vortexed, placed in an ultrasonic bath for 5 min and centrifuged (10 °C, 10 min, 3000 rpm). The organic phase was transferred to clean glass tubes, and the residual aqueous phase of samples was re-extracted with 2 mL of organic phase. Extracts were evaporated to dryness using N_2 stream for 9 min at 30 °C. After this step, 250 μL of a 3:7 acetonitrile: water solution was added. Samples and standards were vortexed, ultrasonicated for 5 min and then filtered into vials. Finally, 2 μL was injected into the LC–MS/HRMS system with a Kinetex XB C18, 2.1 mm × 100 mm (1.7 μm) column (Phenomenex, CA, USA). The mobile phase was a mixture of water (A) and acetonitrile (B) and the flow rate was 150 μL min^{-1}. The elution gradient was (t, %B): (0, 50), (8, 50), (8.1, 100), (10, 100), (10.1, 50), (15, 50).

3. Results and discussion

3.1. Study of MS parameters and flow rate

Systematic experiments were carried out to study the effect of mass spectrometry (MS) parameters, such as automatic gain control (AGC) target, maximum ion time, resolution, scan range, scan events, normalized collision energy (NCE), step NCE and LC column flow rate, on the effective ion time, sensitivity (precursor and product ions) and number of scans per chromatographic peak. Q Exactive offers the possibility to acquire data in different modes (summarized in Table S3, Supplementary Data). Automatic Gain Control (AGC) target and maximum ion time is used to control the number of ions and maximum time taken to fill the C trap, respectively. The ions are accumulated and collisionally damped in C trap (C shaped rf-only quadrupole) before injection into the orbitrap [20]. The most relevant results from the systematic experiments described in Table S2, Supplementary Data are as follows:

- Effective ion time (experiments 1–6), time taken to fill the C trap, which depends on the concentration of the analytes, is very much less (~2 ms) than the maximum injection time (250 ms) for an analyte concentration of 0.1 mg L^{-1} in a standard solution. Therefore, the effective ion time has to be used to calculate the cycle time, rather than the maximum injection time.
- Polarity switching (experiments 10 and 11) takes ~350 ms, which leads to insufficient number of scans/peak. Therefore samples were injected in positive and negative mode separately.
- A UPLC column (100 mm × 2.1 mm, 1.7 μm) with its typical flow rates between 200 and 400 μL min^{-1} and a capillary UPLC column (100 mm × 1 mm, 1.7 μm) with flow rates of 150 and 100 μL min^{-1} were assessed. The decrease in flow rate from 400 μL min^{-1} to 100 μL min^{-1} increased the sensitivity of analytes by a factor of at least 2 (Fig. 1). Although the 1 mm column at low column flow rate provided better sensitivity, the column was shown to be less robust for routine analysis of complex urine

Table 1
Experimental properties of the analytes studied.

	Molecular formula	Precursor ion				Product ion		
		Adduct	Monoisotopic exact mass	Accurate mass	Error (ppm)	Mass followed	Proposed formula	NCE
Trenbolone	$C_{18}H_{22}O_2$	$[M+H]^+$	271.1693	271.1692	0.4	253.1587	$C_{18}H_{21}O^+$	40
17α-Ethinylestradiol	$C_{20}H_{24}O_2$	$[M-H]^-$	295.1704	295.1701	1.0	269.1547	$C_{18}H_{21}O_2^-$	70
Zeranol	$C_{18}H_{26}O_5$	$[M-H]^-$	321.1707	321.1710	0.9	277.1809	$C_{17}H_{25}O_3^-$	40
Stanozolol	$C_{21}H_{32}N_2O$	$[M+H]^+$	329.2587	329.2592	1.5	81.0452	$C_4H_5N_2^+$	80
Dienestrol	$C_{18}H_{18}O_2$	$[M-H]^-$	265.1234	265.1234	0.0	93.0333	$C_6H_5O^-$	55
Diethylstilbestrol	$C_{18}H_{20}O_2$	$[M-H]^-$	267.1391	267.1389	0.7	237.0917	$C_{16}H_{13}O_2^-$	65
Hexestrol	$C_{18}H_{22}O_2$	$[M-H]^-$	269.1547	269.1547	0.0	133.0658	$C_9H_9O^-$	30
Zearalenone	$C_{18}H_{22}O_5$	$[M-H]^-$	317.1394	317.1391	0.9	175.0391	$C_{10}H_7O_3^-$	40
Taleranol	$C_{18}H_{26}O_5$	$[M-H]^-$	321.1707	321.1709	0.6	277.1809	$C_{17}H_{25}O_3^-$	35
Nandrolone D3 (internal standard)	$C_{18}H_{23}O_2D_3$	$[M+H]^+$	278.2193	278.2189	1.4			
Taleranol D4 (internal standard)	$C_{18}H_{22}O_5D_4$	$[M-H]^-$	325.1958	325.1953	1.5			

samples and had a short life span (about 200 injections). In order to have a robust method, a core shell UPLC column with 2.1 mm diameter was chosen for method validation experiments and routine analysis.

3.2. Optimization of acquisition modes

The need for a control laboratory from this kind of full scan hybrid instrument coupled to LC is to monitor the retention time for identification, chromatographic peaks of precursor ion and at least one product ion for quantification and confirmation of an analyte. The acquisition modes were optimized to analyze all nine hormones. In order to obtain a precursor and a product ion, the predefined experiment templates – Full MS/AIF, Full MS/dd MS/MS, tSIM/ddMS/MS and also user defined experiments such as Full MS + tMS/MS can be used.

In Full MS/AIF, each cycle consists of a full MS scan event and an AIF scan event. The first scan event is a full scan of ions (Full MS) in the specified mass range collected in the C trap; in the second scan event (AIF), all ions are fragmented in HCD cell, collected in C trap and analyzed in orbitrap. An Extracted ion chromatogram (XIC) of zeranol and its product ion, acquired by Full MS and AIF scans alternatively, is shown in Figure S1, Supplementary Data. A schematic diagram of a Full MS/AIF cycle acquired at a resolving power of 70,000, corresponding to a transient length of 256 ms in the orbitrap, is shown in Fig. 2.

In Full MS + tMS/MS, the first scan event is a Full MS and the second a tMS/MS, where the precursor specified in the inclusion list is selected by quadrupole, fragmented in HCD cell with specific fragmentation energy and collected in C trap. An isolation width of 1.5 Da was used for the quadrupole. The number of tMS/MS scan events depends directly on the number of precursor ions in the inclusion list eluting at that time. In the inclusion list, the same time window was used for all analytes to simulate co-elution; the scan sequence of a Full MS event and MS/MS events of ions in the inclusion list are shown in Figure S2, Supplementary Data. It can be seen that, in each cycle a Full MS scan event is followed by MS/MS events of each ion in the inclusion list. Although Full MS + tMS/MS is more selective than Full MS/AIF for product ions, the number of data points (scans) per chromatographic peak largely depends on the data in the inclusion list. If there are two co-eluting analytes, the number of scans/peak for a particular product ion is halved. Full MS/dd MS/MS provides the possibility to use the selectivity of quadrupole (MS/MS) while balancing number of scans for products. In Full MS/dd MS/MS, the first scan event is a Full MS and the second and consecutive events (Top *N*) are MS/MS events, which can be triggered by the data acquired in the survey scan. The optimization of data dependent mode is explained in the next section.

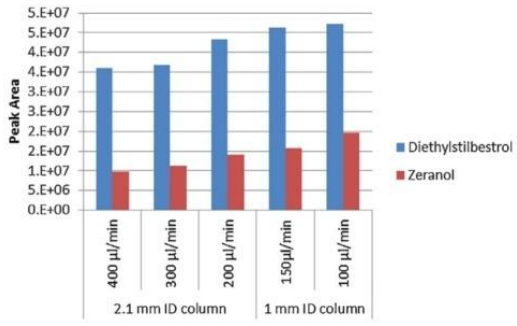

Fig. 1. Effect of LC flow rate on sensitivity of analytes with two different ID (2.1 mm and 1 mm) columns.

3.2.1. Optimization of data dependent acquisition

According to Murray [20], "Data dependent acquisition is a mode of data collection in tandem mass spectrometry in which a fixed number of peaks selected from a survey scan using predetermined rules are selected and the corresponding ions are subjected to MS/MS analysis". As far as we are aware, data dependent acquisition has not been fully evaluated for targeted applications in food safety analysis. A Full MS/dd MS/MS (Top *N*) experiment consists of a full scan event followed by MS/MS scan events of Top *N* precursors in the inclusion list. Figure S3, Supplementary Data shows a Full MS dd MS/MS cycle with the inclusion list containing precursor masses. In each cycle, a Full MS event is followed by MS/MS events of the *N* most abundant ions (3 abundant analytes in case of Fig. 3). A resolving power of 70,000 was used for both Full MS and dd MS/MS events. A Top *N* approach was optimized such that in a given time, when *N* abundant masses in the inclusion list are detected in the survey scan, a dd MS/MS is triggered to select the precursor ion in the inclusion list, fragmented in HCD cell, collected in the C trap and analyzed in the orbitrap. Compared to the Full MS/AIF, the use of an inclusion list with the monoisotopic mass and specific NCEs of the precursors provided sensitive products as precursors are selected in the quadrupole. The time window was given over the whole chromatographic method, which blinds the retention time parameter in the list. In Top *N*, *N* depends on the loop count and multiplex (*N* = loop count × multiplex). In this study, multiplex for product ion scan was not assessed, and hence *N* corresponds directly to loop count (multiplex was set as 1). Thus, in Top 1 the maximum number of dd MS/MS which can be triggered is 1, which is the most abundant among the masses in the inclusion list at a particular retention time; similarly for Top *N*, *N* dd MS/MS will be triggered. So, the co-eluting analytes limit the number of MS/MS scans. The effect of loop count on co-eluting peaks is shown in Fig. 4, which shows the comparison of loop count 1 and loop

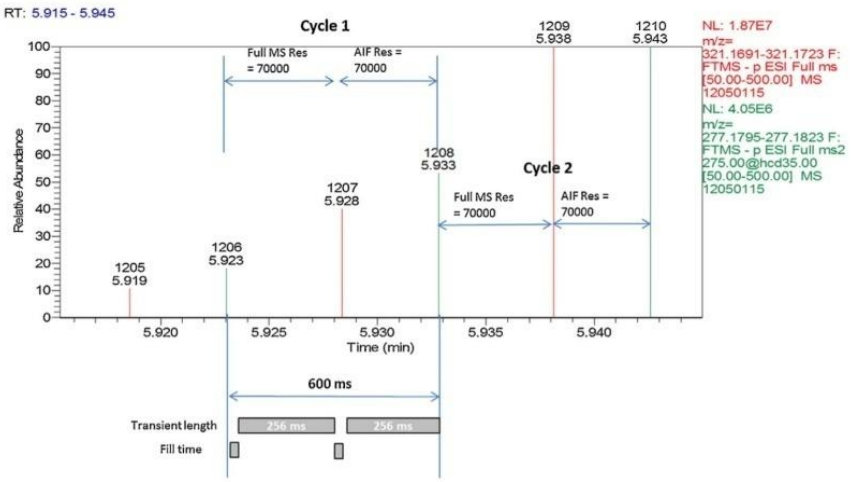

Fig. 2. Schematic of a Full MS/AIF cycle (resolution – 70,000/70,000) experiment.

count 2 with two co-eluting peaks. In a Top 1 (loop count 1, msx 1) MS/MS experiment, MS/MS is triggered for the most abundant ion at any time. Thus, when two analytes co-elute, a Top 2 method is necessary to trigger an MS/MS experiment on both analytes. Moreover, the loop count influences linearly the number of data points per peak: Fig. 5 shows that Top 2 (loop count 2) has half as many data points per chromatographic peak as Top 1 (loop count 1). The data dependent settings (under fill ratio, apex trigger, charge exclusion, dynamic exclusion, exclude isotopes and peptide match) were not used, as these settings exclude MS/MS scans. The data dependent approach used in this work is more suitably described as exact mass dependent acquisition, since it depends only on exact mass in the inclusion list to trigger dd MS/MS, and fragmentation is carried out with the NCE specified in the inclusion list. This approach

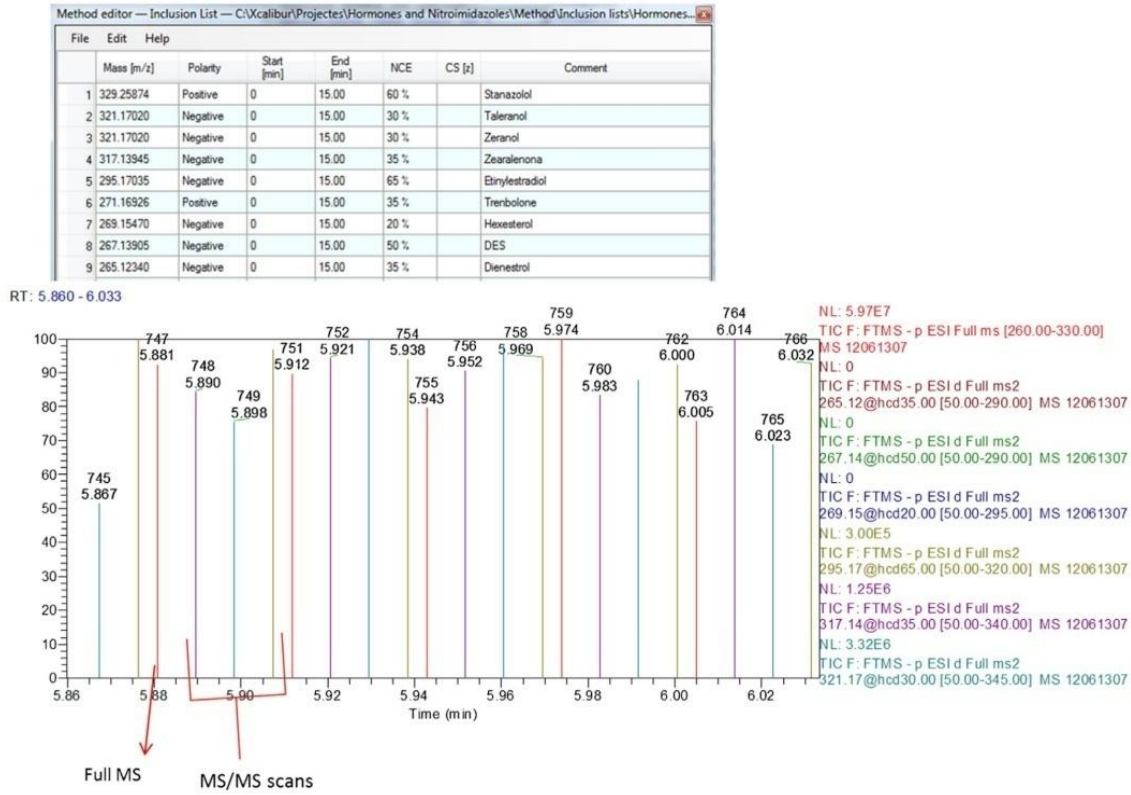

Fig. 3. A cycle of Full MS/dd MS/MS (Top 3) experiment. It can be seen that in each cycle, a Full MS event is followed by Top 3 MS/MS events of the masses in the inclusion list. In the inclusion list, the retention time is set to the whole chromatographic period (0–15 min) which allows to blind the retention time parameter during dd MS/MS scans.

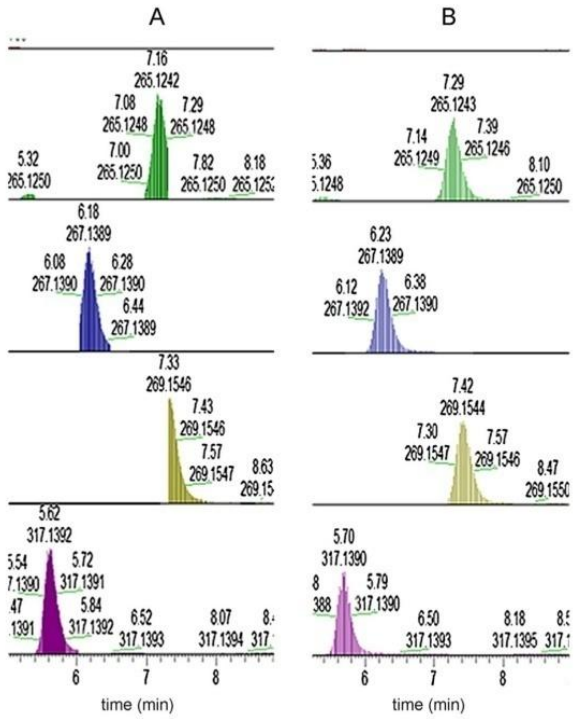

Fig. 4. Effect of loop count on scans per peak and co-eluting peaks: loop count 1 (A) loop count 2 (B).

is more advantageous than Full MS + tMS/MS as it does not depend on the retention time of the analyte, especially when there is a need to analyze suspected compounds for which the method is not validated. Also, this approach improves the number of scans per peak of the product ions and triggers MS/MS based on abundance, compared to Full MS + tMS/MS. As an example, a product ion spectrum of trenbolone and the chromatographic profiles of precursor and product ions are shown as insets in Figure S3, Supplementary Data.

When complex matrices such as urine are analyzed, post interface suppression of analyte signal can occur due to matrix interferences [21], leading to low sensitivity. In the case of matrix-matched standards, the sensitivity of analytes was low in all modes studied up to now. In order to avoid the signal suppression, intermediate collision energy was used to extract the precursor ion from the ddMS/MS filter (product ion spectrum). This provided the possibility to use quadrupole in MS and MS/MS scans. Further, time and mass functions were created to reduce the ions in the C trap during the Full MS as much as possible by reducing the mass range over the retention time of the analyte (minimum for full scan is 10.1 Da). This approach increases the signal to noise ratio of the analytes, thereby having better detection limits for the analytes. Choosing the precursor ion from the quadrupole selected dd MS/MS filter also increases the signal to noise ratio which is evident at low concentrations (Fig. 6). Therefore, the precursor ion peak can be used for quantification and the product ion peak (complete chromatographic profile) or product ion spectrum can be used for confirmation. Also the Full MS scans provide the possibility to use the isotopic pattern for additional confirmation. The optimized Full MS dd MS/MS settings are shown in Figure S4, Supplementary Data, where retention time is set to the whole time window. Although Full MS/ddMS/MS uses quadrupole for MS/MS scans, quadrupole cannot be used for the Full MS scan less than 10.1 Da range.

In order to obtain quadrupole-selected precursor ions without being fragmented, we assessed tSIM/ddMS/MS. In tSIM/ddMS/MS, the precursor in the inclusion list (with retention times) can be selected in the quadrupole with a particular isolation width, followed by a ddMS/MS scan. The number of precursor scans for co-eluting analytes can be augmented by multiplex (msx). The precursor ions and products were acquired using tSIM (loop count 2, msx 2) and ddMS/MS (loop count 3, msx 1) and are shown in Figure S5, Supplementary Data. The settings of the tSIM/ddMS/MS experiment are shown in Figure S6, Supplementary Data. The signal to noise (S/N) ratios of trenbolone precursor in different modes acquired in different mass ranges at 1 $\mu g\,L^{-1}$ in matrix are shown in Fig. 7. This figure highlights the fact that a narrower mass range in Full MS (10.1 Da) and tSIM modes using the quadrupole increases the S/N ratio considerably, which leads to better method detection limits. Figure S7, Supplementary Data shows the trenbolone product ion (253.1587) in a spiked urine sample at a concentration of 1 $\mu g\,L^{-1}$ acquired with different modes, where the S/N ratio is higher in all modes using quadrupole; in AIF, where quadrupole is not used, no peak is seen. The method features of the various modes are summarized in Table 3.

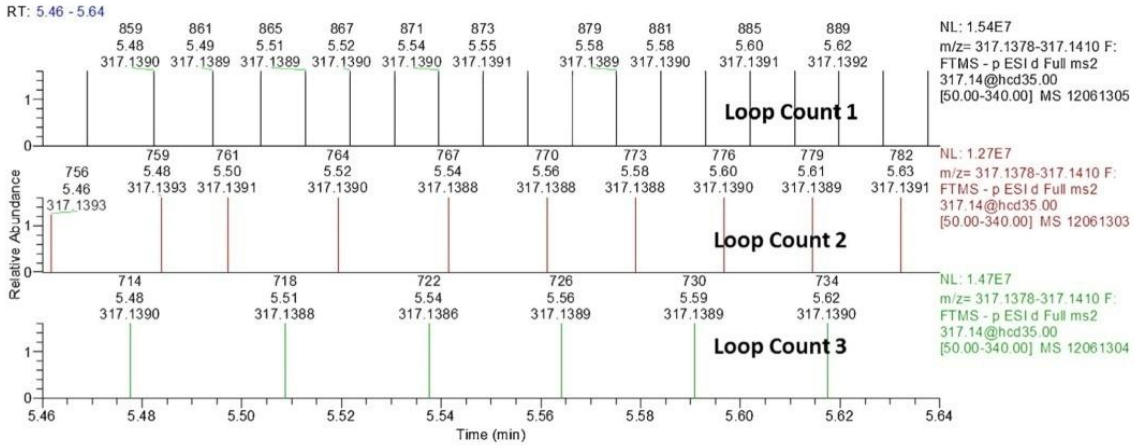

Fig. 5. A XIC (stick display) of zearalenone (317.1394) acquired with three different loop count settings showing the effect of loop count on scans/peak. In loop count 3, MS/MS data points are acquired every 3rd scan (Top 3 approach).

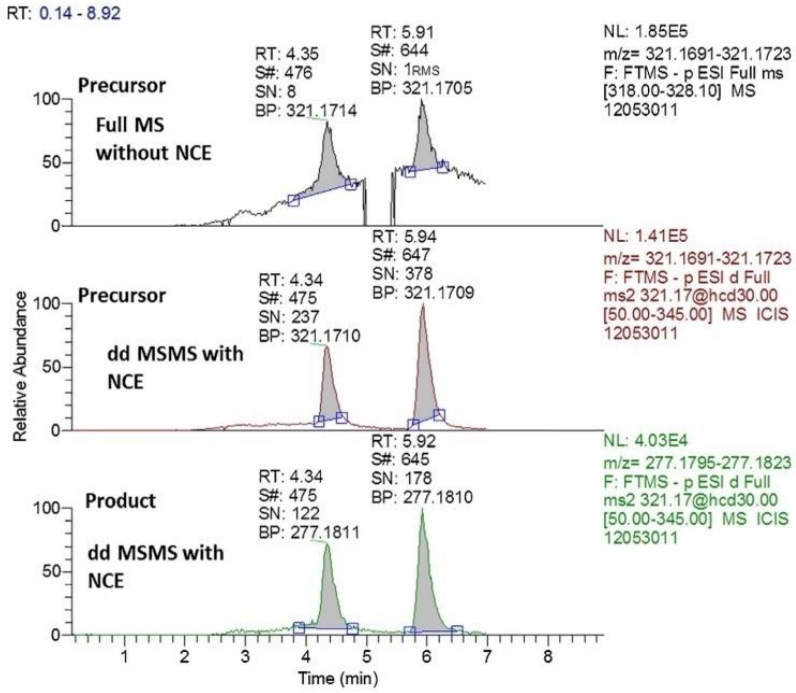

Fig. 6. Taleranol and zeranol at 1 μg L^{-1} in urine sample. Precursor in dd MS/MS filter has higher signal to noise ratio than the precursor in full MS filter.

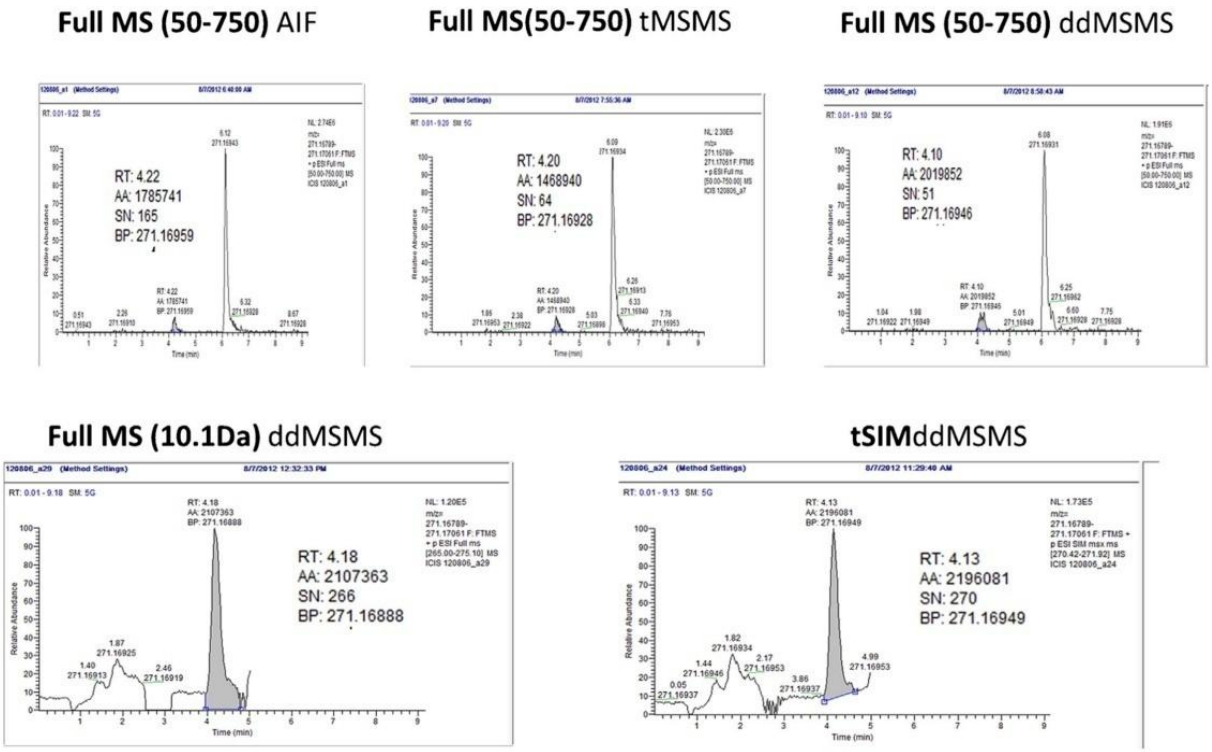

Fig. 7. Trenbolone (precursor ion – 271.1693) in a spiked sample at a concentration of 1 μg L^{-1} in urine acquired with different modes, showing the increased signal-to-noise ratio (SN) with targeted modes (Full MS 10.1 Da and tSIM).

Table 2
Validation data.

Analyte	Extraction recoveries%[a]	Calibration equation (y=)	CC alpha ($\mu g\,L^{-1}$)	CC beta ($\mu g\,L^{-1}$)	Within laboratory reproducibility (n = 9 days) (RSD%) at four levels				Trueness (error%)[a]
					Level 1 (1 $\mu g\,L^{-1}$)	Level 2 (2 $\mu g\,L^{-1}$)	Level 3 (10 $\mu g\,L^{-1}$)	Level 4 (20 $\mu g\,L^{-1}$)	
Trenbolone	87	0.007 + 0.515x	0.16	0.33	10.0	4.6	3.5	0.9	2.3
17α-Ethinylestradiol	80	−0.001 + 0.003x	0.42	0.63	12.6	7.4	6.8	1.6	1.7
Zeranol	83	−0.005 + 0.097x	0.35	0.51	10.2	4.9	4.9	1.2	1.2
Stanozolol	65	−0.185 + 0.623x	0.69	0.90	13.1	14.9	4.7	1.2	1.4
Dienestrol	74	−0.006 + 0.017x	0.11	0.29	11.0	9.4	10.0	1.2	1.9
Diethylstilbestrol	71	−0.003 + 0.012x	0.43	0.50	4.9	11.9	4.7	1.1	2.0
Hexestrol	75	−0.0004 + 0.007x	0.25	0.42	10.9	8.5	11.7	0.8	3.0
Zearalenone	79	−0.002 + 0.033x	0.38	0.55	9.9	8.4	3.7	0.6	1.4
Taleranol	83	0.032 + 0.069x	0.49	0.80	18.2	4.9	7.4	0.7	2.9

[a] Average of four concentration levels – 1 $\mu g\,L^{-1}$, 2 $\mu g\,L^{-1}$, 10 $\mu g\,L^{-1}$, 20 $\mu g\,L^{-1}$.

Table 3
Different acquisition modes and their features.

Acquisition modes	Compound specific NCE	Peak area (sensitivity)[a]		Signal to noise[a]		No of scans/peak[a]		Full scan data
		Precursors	Products	Precursors	Products	Precursors	Products	
Full MS (50–750) AIF	No	++	+	+	+	+	+	Yes
Full MS (50–750) tMS/MS	Yes	++	+++	+	+++	+	+++	Yes for precursors
Full MS (50–750) dd MS/MS	Yes	++	+++	+	+++	+	+++	Yes for precursors
Full MS (10.1 Da) dd MS/MS	Yes	++	+++	+++	+++	+++	+++	No
tSIMddMS/MS	Yes	++	+++	+++	+++	+++	+++	No

[a] Based on low concentration levels (0.3 $\mu g\,L^{-1}$ and 1 $\mu g\,L^{-1}$) in sample.

3.3. Method validation and application to real samples

The optimized method with tSIM/ddMS/MS as acquisition mode was validated according to Commission Decision 2002/657/EC. The parameters evaluated were extraction recovery, specificity, linearity, precision, trueness, CCα and CCβ. The results of this validation are summarized in Table 2. Extraction recoveries were assessed by spiking blank urine sample before and after extraction at four concentration levels with five replicates at each level. Specificity was assessed by verifying the presence of interference at the retention time of analytes greater than a signal-to-noise ratio of three. Linearity was assessed with spiked blank matrix at four concentration levels ranging between 1 and 20 $\mu g\,L^{-1}$. For all analytes, r^2 was greater than 0.99 and the deviation of each point from the calibration line was lower than 15%. Within-laboratory reproducibility was assessed by spiking blank urine samples at four different concentrations on nine different days. Trueness was calculated as the percentage of error between spiked and found concentrations. CCα was estimated from the calibration curve prepared by spiking blank matrices at four concentration levels in the low concentration range. CCα is calculated as the concentration corresponding to the y-intercept plus 2.33 times its standard deviation. In the case of CCβ, the concentration corresponds to CCα + 1.64s, s being the standard deviation obtained at the CCα level. CCα ranged between 0.11 $\mu g\,L^{-1}$ and 0.69 $\mu g\,L^{-1}$, and CCβ ranged between 0.29 $\mu g\,L^{-1}$ and 0.90 $\mu g\,L^{-1}$. Limits of detection (LOD) and quantification (LOQ) were estimated as three and ten times, respectively, the signal to noise ratio. Values obtained ranged between 0.002 $\mu g\,L^{-1}$ and 0.03 $\mu g\,L^{-1}$ for LOD, and between 0.008 $\mu g\,L^{-1}$ and 0.1 $\mu g\,L^{-1}$ for LOQ. As frequently happens in MS, this approach leads to unrealistically low values. In this scenario, for a forbidden substance the CCα value can be considered a more realistic estimation of the LOD.

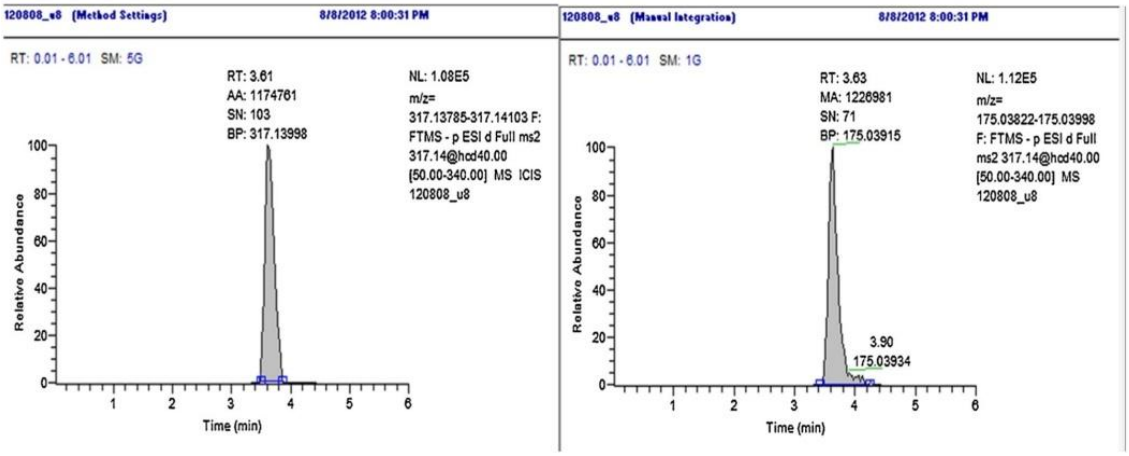

Fig. 8. A XIC of a positive sample with zearalenone (precursor – 317.1394, product – 175.0391) at 1.177 $\mu g\,L^{-1}$ concentration.

On the other hand, the minimum concentration in matrix at which the analytes are confirmed and quantified (i.e. 1 $\mu g\,L^{-1}$), and which was set as the lowest value of the calibration curves, is a realistic estimation of LOQ.

The CCα values of the proposed LC–MS/HRMS method are comparable to the limits of the previously reported LC–MS/MS methods, summarized in Table S4, Supplementary Data. The developed method is being used for the routine analysis of real samples and to date one positive urine sample has been found for zearalenone (Fig. 8).

4. Conclusions

Evaluation experiments showed that polarity switching reduces the instrument's scan speed for acquiring precursors and products at a resolving power of 70,000, thereby dramatically reducing the number of scans per chromatographic peak. Therefore, samples are injected in positive and negative mode, separately. Although Q Exactive uses collision energies normalized to the masses, it was seen that optimal NCE varies between analytes in the case of hormones.

The decrease in mass range in Full MS or tSIM modes increases the signal to noise ratio of the analytes, thereby leading to better detection limits.

The data dependent acquisition (Top *N*) approach was optimized in a way that uses compound-specific collision energies and triggers ddMS/MS when the masses specified in the inclusion list are detected in the survey scan in case of Full MS/ddMS/MS, or the inclusion list with retention time, masses and NCEs in case of tSIM/ddMS/MS. In Full MS/ddMS/MS, precursors and products can be acquired from MS/MS experiments when intermediate collision energy is used and are independent of retention time. The limitation for MS/MS scans is the number of co-eluting analytes, which increases the *N* in Top *N* and eventually reduces the number of scans per peak by *N* times. The optimized tSIM/ddMS/MS method gives good quantitation parameters for the analysis of synthetic hormones in urine and the sensitivity similar to LC–MS/MS have been achieved. The presence of quadrupole helps to reduce the post interface ion suppression in the C trap, which is a critical issue in case of complex matrices.

In this sense, the combination of quadrupole with the orbitrap is extremely interesting, because in addition to the excellent performance of the mass analyzer, sensitivity and robustness is improved in the analysis of low concentration levels in the presence of highly complex matrices, as shown in the application presented in this paper. Q Exactive has been shown to be a trustworthy alternative to triple quadrupole instruments.

Finally, this new and powerful equipment, after the improvement of the software for data mining, could offer new and exciting insights in the scarcely explored field of non target analysis of residues.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgments

This study was supported by the Spanish Ministerio de Ciencia e Innovacion (Project AGL 2008-05578-C05-03). P. Kumar thanks the Generalitat de Catalunya for a FI grant (Grant Number 2012 FI-B2 00217). The authors thank Thermo Scientific for technical support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2013.04.017.

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Supplementary Data for

Targeted analysis with bench top quadrupole - Orbitrap hybrid mass spectrometer: Application to determination of synthetic hormones in animal urine

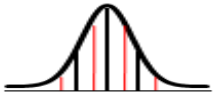
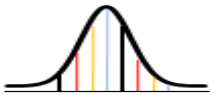
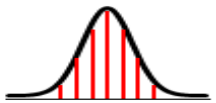
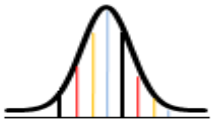
Table S1. Requirements for relative ion intensities (sample compared to standard) in mass spectrometric techniques as in Codex Alimentarius Commission guidelines document.

Relative ion intensity (% of base peak)	**Tolerances for ion ratio LC-MS, LC-MS/MS (relative)**
(%)	(%)
> 50	≤20
20-50	≤25
>10 - < 20	≤30
≤10	≤50

Table S2. Design of experiments to study different parameters in Q Exactive with model analytes – Diethylstilbestrol, stanazolol and zeranol at a concentration of 0.1 mg L^{-1}

Parameters	Experiments	Factors						
		AGC Target (prec,pdt)	Maximum Ion time (prec/ pdt) ms	Resolution (prec/pdt)	Scan Range (prec/pdt)	Scan Events	NCE	Step NCE
Ion time	1	1 e 6	2	35000	50-500	Full MS/AIF_POS and NEG	35	
	2	1 e 6	10	35000	50-500	Full MS/AIF_POS and NEG	35	
	3	1 e 6	60	35000	50-500	Full MS/AIF_POS and NEG	35	
	4	1 e 6	120	35000	50-500	Full MS/AIF_POS and NEG	35	
	5	1 e 6	250	35000	50-500	Full MS/AIF_POS and NEG	35	
	6	1 e 6	500	35000	50-500	Full MS/AIF_POS and NEG	35	
Resolution	7	1 e 6	250	17500	50-500	Full MS/AIF_POS and NEG	35	
	8	1 e 6	250	35000	50-500	Full MS/AIF_POS and NEG	35	
	9	1 e 6	250	70000	50-500	Full MS/AIF_POS and NEG	35	
	9a	2e 4	250	70000	50-500	Full MS/AIF_POS and NEG	35	
Polarity Switching	10	1 e 6	250	35000	50-500	Full MS/AIF_POS and NEG	35	
	11	1 e 6	250	35000	50-500	Full MS/AIF_POS	35	
	12	1 e 6	250	35000	50-500	Full MS/AIF_NEG	35	
Res	13	1 e 6	250	70000	50-500	Full MS/AIF_NEG	35	
	14	1 e 6	250	140000	50-500	Full MS/AIF_NEG	35	
Scan Range	15	1 e 6	250	35000	50-500	Full MS/AIF_POS and NEG	35	
	16	1 e 6	250	35000	75-1000	Full MS/AIF_POS and NEG	35	
	17	1 e 6	250	35000	50-500	Full MS/AIF_POS	35	
	18	1 e 6	250	35000	75-1000	Full MS/AIF_POS	35	
Step NCE	19	1 e 6	250	35000	50-500	Full MS/AIF_POS and NEG	50	30
tMS/MS	20	1 e 6/2e5	250 /120	35000	50-500	Full MS+t MS/MS_msx_1 (NEG)		

Table S3. Acquisition experiments (templates) and their features.

Template	Quadrupole	HCD cell	Retention time dependent	Scan Sequence	Description
Full MS	No	No	No		Full MS scan of the precursor ions
Full MS/AIF	No/No	No/Yes	No		Full MS scan of precursor ions and full MS scan of product ions (normalized collision energy in HCD cell).
Full MS/dd-MS^2 (TOP N)	No/Yes	No/Yes	Yes or No		Full MS scan of precursor ions and N MS/MS scans of precursor ions selected from an inclusion list. An example of Top 3 ddMS/MS with 5 ions coeluting.
Targeted SIM	Yes	No	Yes		Ions from an inclusion list filtered in the quadrupole at a specified isolation width.
Targeted MS^2	Yes	Yes	Yes		Ions from an inclusion list filtered in the quadrupole at a specified isolation width. Full scan of product ions.
Targeted SIM/dd-MS^2 (TOP N)	Yes/Yes	No/Yes	Yes		Targeted SIM scan of precursor ions and N MS/MS scans of precursor ions selected from an inclusion list.

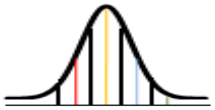
					An example of Top 3 ddMS/MS with 5 ions coeluting.
Full MS+tMS/MS	No/Yes	No/Yes	Yes		An user defined template with Full MS scan of precursor ions and MS/MS scans of precursor ions selected from an inclusion list. An example of Top 3 ddMS/MS with 5 ions coeluting.

Table S4. Selected methods for analysis of synthetic hormones in urine.

Sl.No.	Analytes	Matrix	Detector	Limits	Reference
1	17α-trenbolone 6-β-OH-stanozolol	urine	LC MS MS	CCα - 1,44 ng mL^{-1} CCα - 3,77 ng mL^{-1}	Pouke, 2002
2	16β-Hydroxystanozolol, α-Trenbolone, Diethylstilbestrol, Dienestrol, Hexestrol α-Zeranol, β-Zeranol and Ethinylestradiol	bovine urine	LC MS MS	CCα – between 0,44 and 1.07 ng mL^{-1}	Pouke, 2005
3	Stanozolol	human urine	LC MS MS	LOD - 0,1 ng mL^{-1}	M.Thevis, 2006

4	Stanozolol, taleranol, zeranol, hexestrol, dienestrol, ethynylestradiol, diethylstilbestrol, and trenbolone	Urine	LC MS MS	CCα – between 0.2 and 0.9 ng mL^{-1}	A.Rubies, 2007
5	Stanozolol, taleranol, zeranol, hexestrol, dienestrol, ethynylestradiol, diethylstilbestrol, trenbolone and zearalenone.	Urine	LC MS HRMS	CCα between 0.11 ng mL^{-1} and 0.69 ng mL^{-1}	Current article

CCα – Decision limit, LOD – Limit of detection.

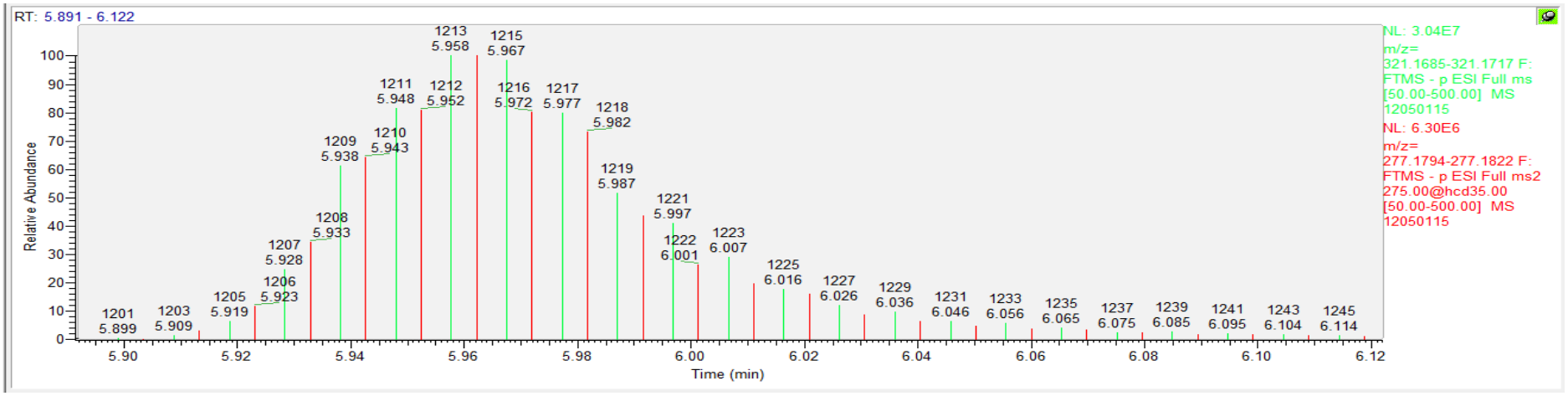


Figure S1. Extracted ion Chromatographic peak (stick display) of Zeranol and its fragment acquired in Full MS/AIF (overlaid). Above each peak are the retention time and scan number. It can be seen that Full MS and AIF scan events alternatively.

	Mass [m/z]	Polarity	Start [min]	End [min]	NCE	CS [z]	Comment
1	271.16926	Positive	5.00	9.00	40 %		Trenbolone
2	295.17035	Negative	5.00	9.00	70 %		Etinylestradiol
3	321.17020	Negative	5.00	9.00	35 %		Zeranol
4	329.25874	Positive	5.00	9.00	80 %		Stanazolol
5	265.12340	Negative	5.00	9.00	50 %		Dienestrol
6	267.13905	Negative	5.00	9.00	65 %		DES
7	269.15470	Negative	5.00	9.00	30 %		Hexesterol
8	317.13945	Negative	5.00	9.00	40 %		Zearalenona
9	321.17020	Negative	5.00	9.00	35 %		Taleranol

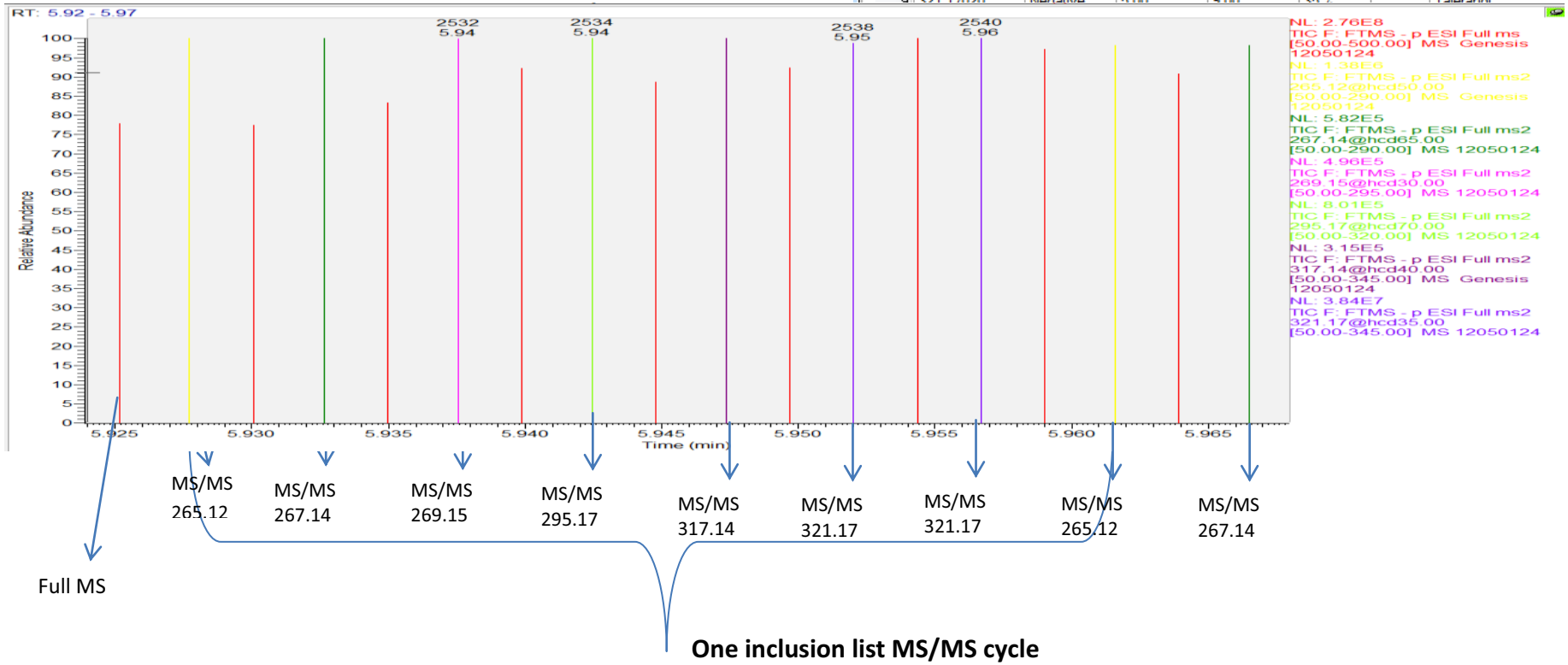


Figure S2. A cycle of Full MS + tMS/MS (Res 35000/35000) with the inclusion list masses. Retention time has been set to be the same. It can be seen that Full MS and MS/MS events of all the masses in inclusion list are carried out alternatively.

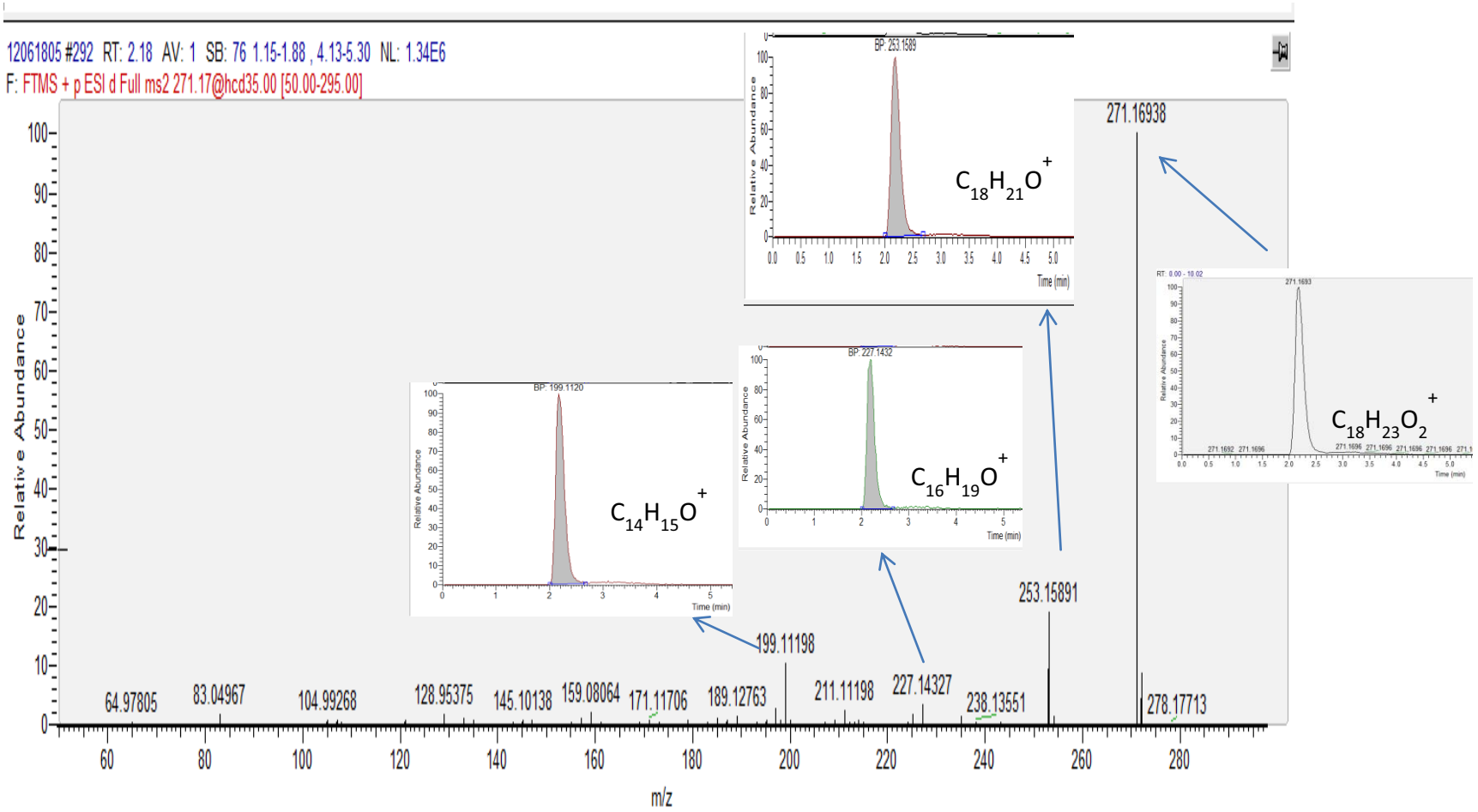


Figure S3. A product ion spectrum of trenbolone acquired in Full MS/dd MS/MS mode. The chromatographic profiles of precursor ions and product ions are shown in insets

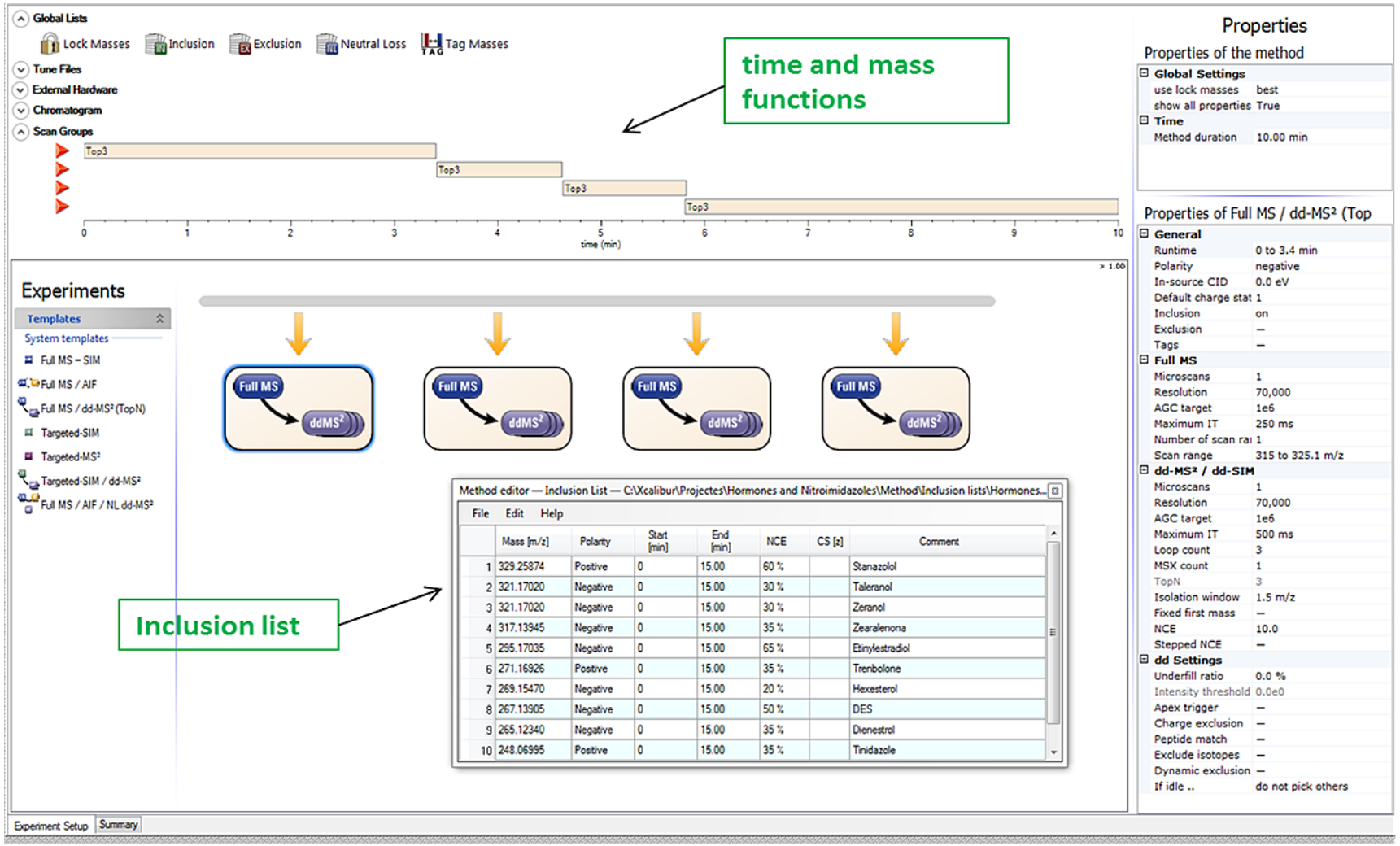


Figure S4. Full MS/dd MS/MS settings in negative mode, where RT is set to whole chromatographic time.

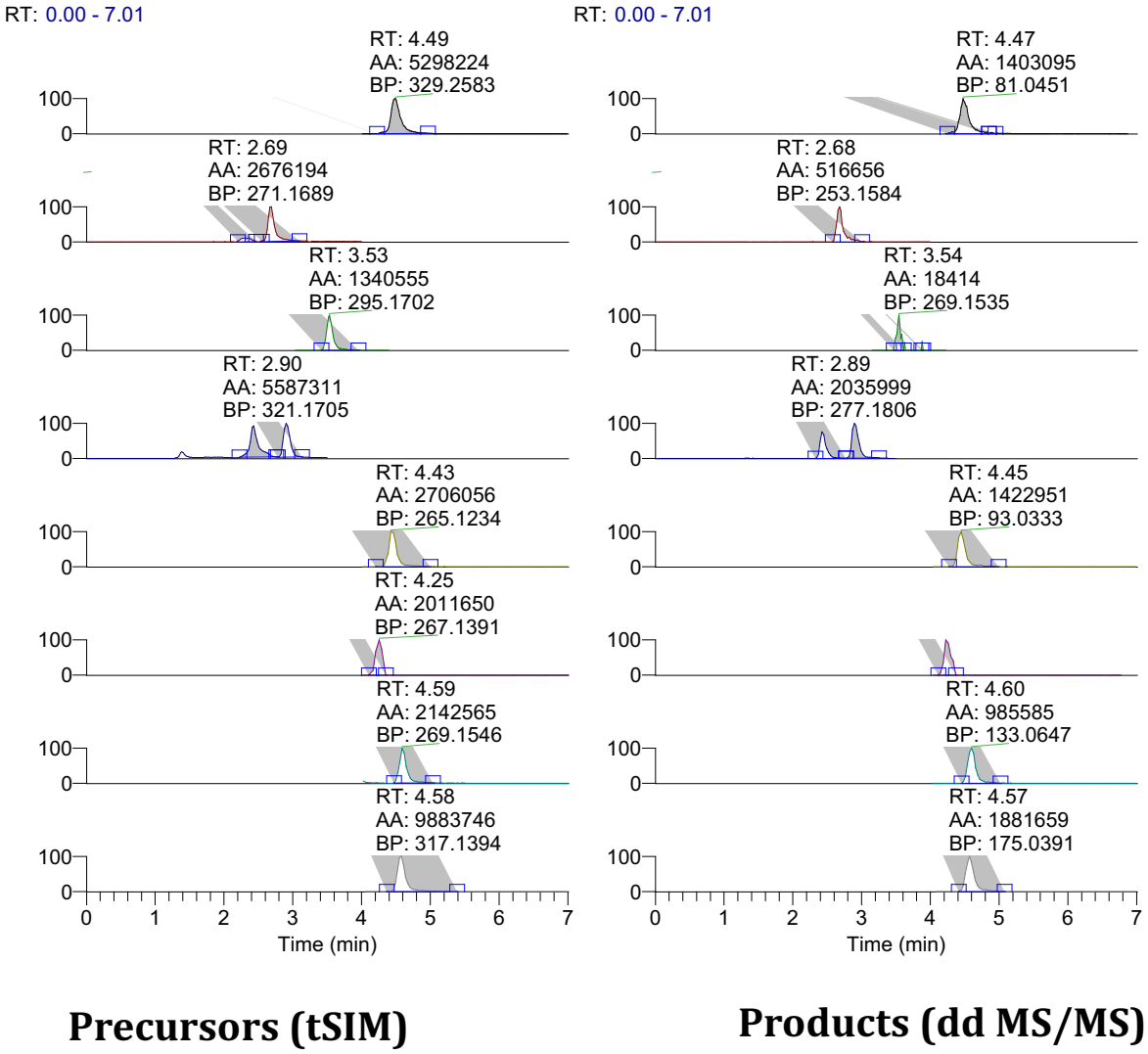


Figure S5. A chromatogram of a blank urine sample spiked with the studied hormones at a concentration of 1 µg L^{-1}. Acquisition with tsim (loopcount 2 msx2) dd MS/MS (loopcount3 msx1) with an isolation width of 1.5 Da.

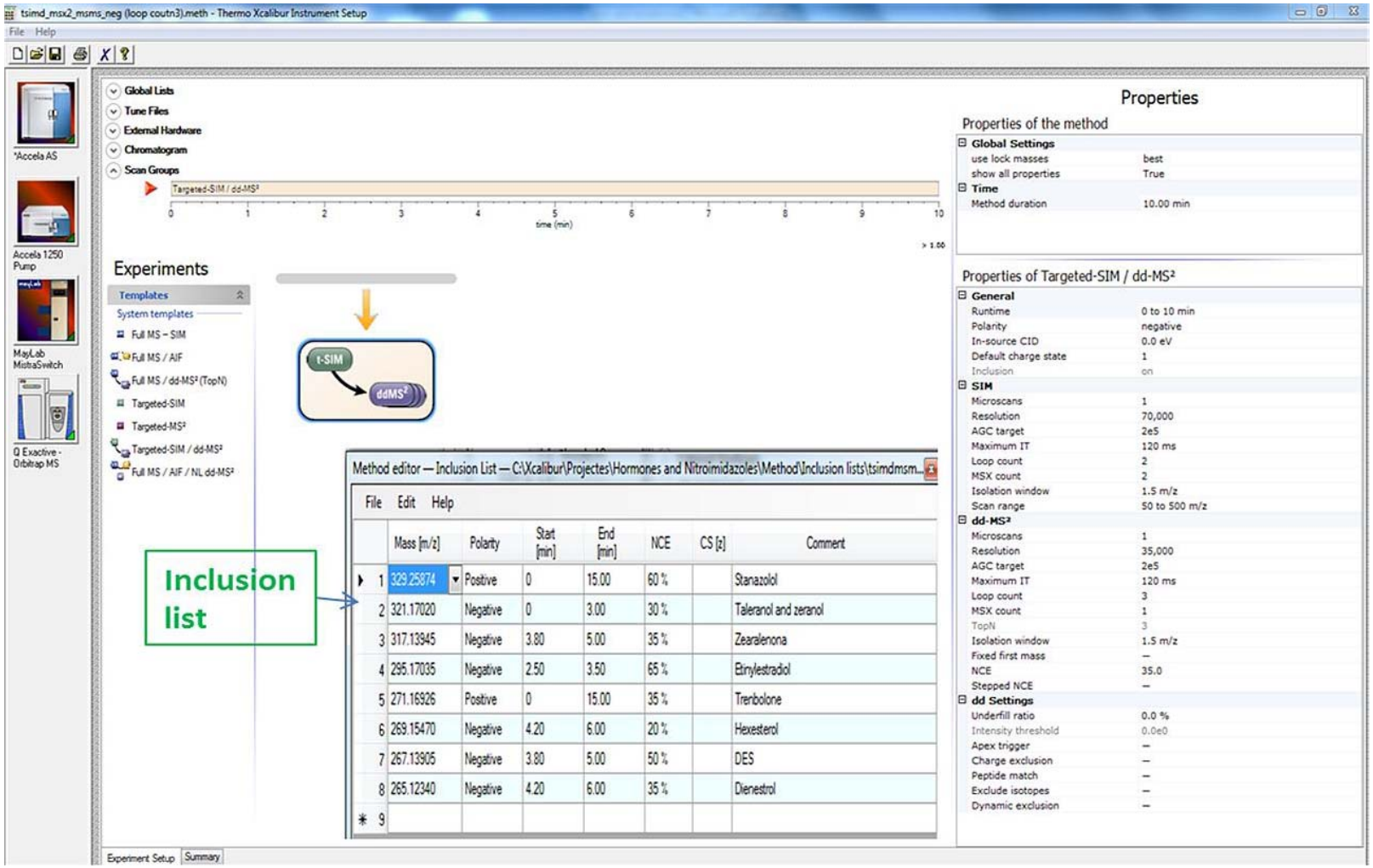
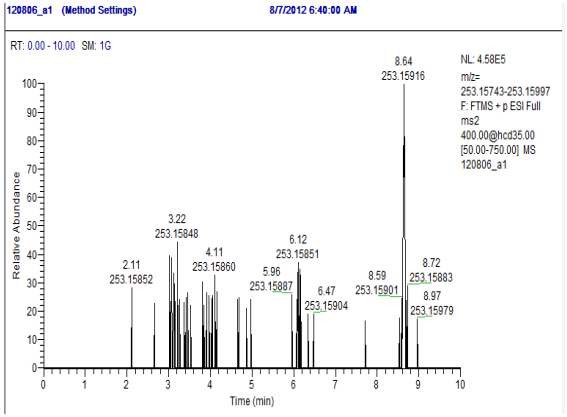
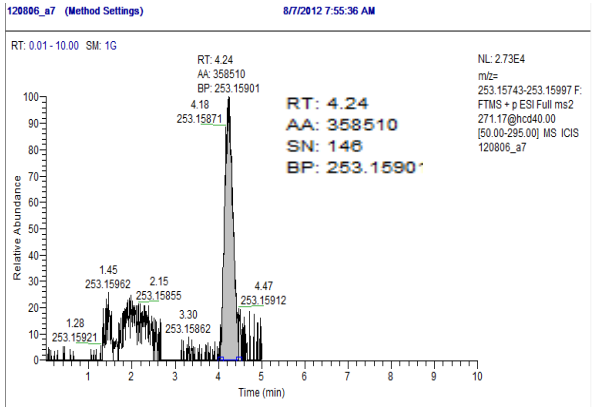


Figure S6. The settings of tSIM/ddMS/MS method in negative mode.

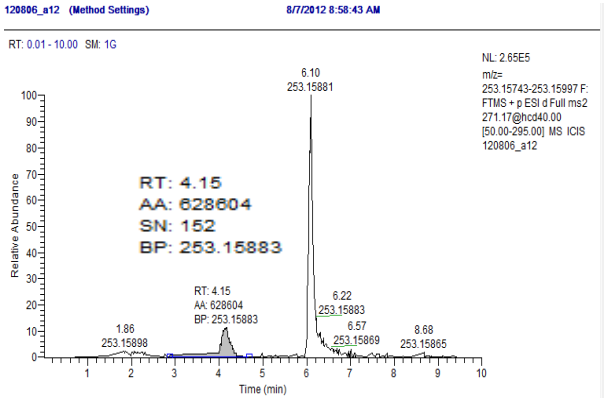
Full MS **AIF**



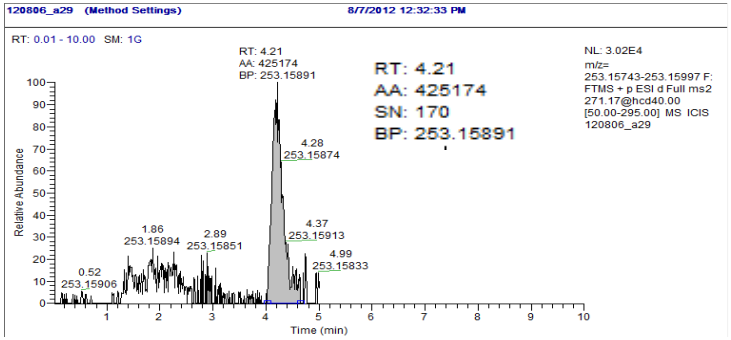
Full MS **tMSMS**



Full MS (50-750) **ddMSMS**



Full MS (10.1Da) **ddMSMS**



tSIM **ddMSMS**

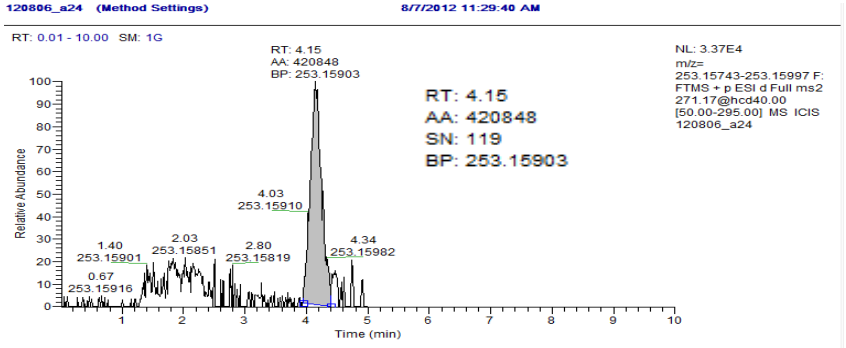


Figure S7. Trenbolone (product ion – 253.1587) in a spiked sample at a concentration of 1 µg/kg in urine acquired with different modes, where the signal-to-noise ratio (S/N) can be compared.

6.3. Discussion

A hybrid mass spectrometer is a device for tandem mass spectrometry that consists of a combination of two or more m/z separation devices of different types [130]. Hybrid mass spectrometers are constructed with the goal of combining different performance characteristics of various analyzers into one instrument and achieve higher performance [131]. The Q-Orbitrap hybrid mass spectrometer, combines the quadrupole mass filter with the high resolving power Orbitrap analyzer. Performance of hybrid mass spectrometers can be measured with three metrics : sensitivity, resolving power and production rate & richness of dataset [132]. The Q-Orbitrap instrument provides the possibility to analyze in Full Scan mode by using quadrupole for ion transmission alone and also analyze in targeted modes of acquisition by using quadrupole at specific mass isolation widths, up to 0.4 Da (Figure 6.2). In this study it was observed that apart from matrix ion suppression which is common in the ionization source, post interface ion suppression occur and it can be mitigated by using quadrupole filter. Kaufmann et. al. [57] first observed such post interface suppression due to matrix ions in single stage Orbitrap instrument and proposed it to occur in C trap.

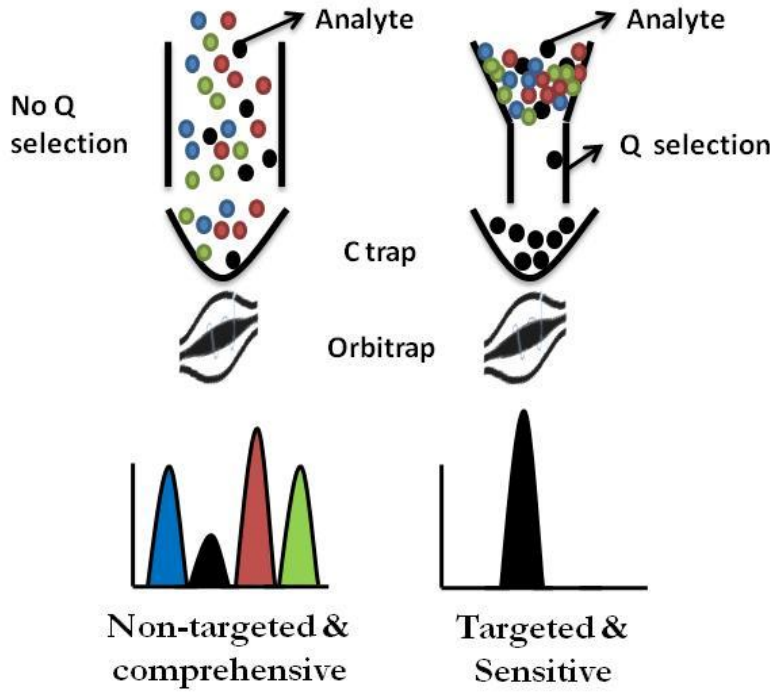
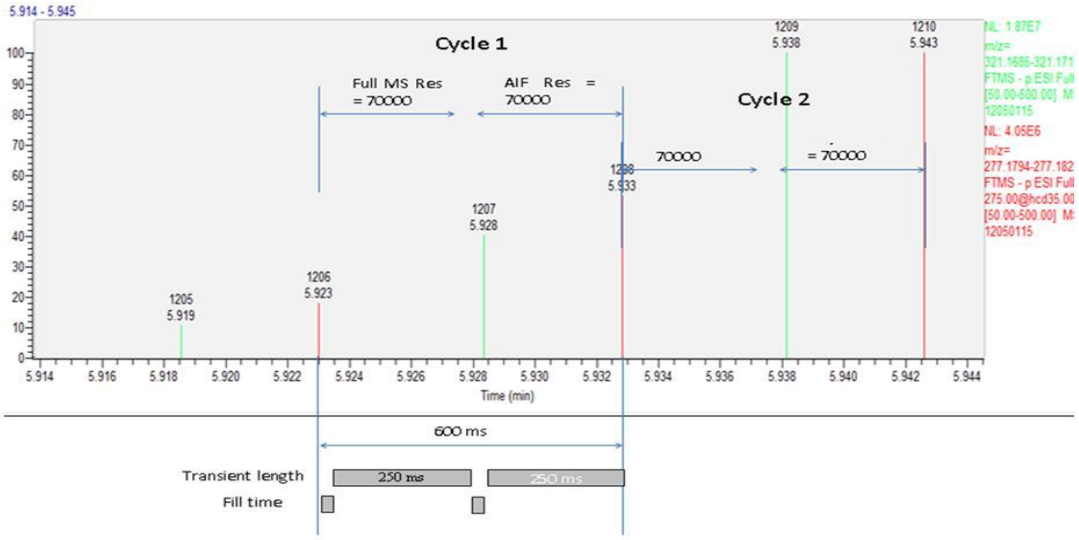


Figure 6.2. The presence of quadrupole as a mass filter in Q-Orbitrap mass spectrometer.

The application of the Q-Orbitrap mass spectrometer coupled to liquid chromatography to analyze synthetic hormones in animal urine gave satisfactory sensitivity (first metric) with targeted modes of acquisition. The other figures of merit from validation studies are summarized in table 6.4.

Table 6.4. Performance characteristics of the method from validation studies for the analysis of synthetic hormones in urine.

CCα	0.11 – 0.69 µg/l
CCβ	0.33 – 0.90 µg/l
Trueness (as bias)	1.4 – 3 %
Interday Precision at low concentrations (1 µg/l)	4.9 – 18.2 %
Extraction recoveries	65 – 83 %



Mass Resolution at m/z 200	Transient Length (ms) in orbitrap	Scan Rate (scans/sec)
17500	64	12
35000	128	7
70000	256	3
140000	512	1.5

Number of scans per 7 seconds chromatographic peak = 7000 ms/600 ms = atleast 11 scans per peak

Figure 6.3. Duty cycle of a Full MS/AIF acquisition cycle in a chromatographic peak of zeranol standard solution.

The Orbitrap mass analyzer provides resolving power (second metric) of up to 140,000 FWHM at 200 m/z which depends on the transient length of ions in the Orbitrap. Apart from high resolving power, the instrument has the capacity to provide accurate mass measurements even less than 2 ppm with external calibration for small molecules with molecular mass lower than 1000 Da.

The third metric, duty cycle in this equipment varies for different acquisition modes. Mainly, duty cycle depends on the resolving power chosen. As shown in Figure 6.3, in a Full MS/AIF experiment, with a 70,000 FWHM resolving power chosen, each cycle comprising one Full MS scan event and AIF scan event takes 600 milliseconds. This is relatively high compared to a TOF instrument where duty cycle is in the order of microseconds [132]. In a chromatographic peak of 7 seconds, 11 Full MS scans can be attained. Moreover, hybrid configuration provide the possibility to attain data dependent acquisition which can trigger MS/MS experiments by taking decisions "on the flight" based on intensity and/or information from an inclusion list of precursor ion masses. Depending on the mode of acquisition, the size of the data files increase ranging from 5 MB to hundreds of MB.

The MS/HRMS system provide required sensitivity using the quadrupole modes of acquisition and moreover the full scan capability provide the possibility to retrospectively analyze the data for non targeted analysis, for which there is a high need in food safety testing.

7. A false positive case due to matrix interference in the analysis of ronidazole residues in muscle tissue (ARTICLE V)

Meat Science 97 (2014) 214–219

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

A false positive case due to matrix interference in the analysis of ronidazole residues in muscle tissue using LC–MS/MS

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ARTICLE INFO

Article history:
Received 12 September 2013
Received in revised form 31 January 2014
Accepted 2 February 2014
Available online 10 February 2014

Keywords:
Ronidazole
Muscle
High resolution mass spectrometry
Quadrupole–Orbitrap
False positive case

ABSTRACT

In contrast with the information of the inspection body concerning the use of ronidazole, several non compliant muscle samples were detected using a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method in accordance with confirmation criteria of Decision 2002/657/EC. This led to the suspicion that non compliance could be due to false positive results. In this context, a liquid chromatography–high resolution mass spectrometry (LC–HRMS) method was developed and sample extracts were re-analyzed, resolving the co eluting isobaric interfering peak, which also has an interfering product ion with the transition product (m/z 201 > 140).

1. Introduction

In most food testing laboratories, the triple quadrupole mass analyzer is the most commonly used instrument for confirming the presence of contaminants in samples. However, the low resolution of the triple quadrupole is not always sufficient for resolving co-eluting isobaric interferences in complex food matrices, thus affecting the reliability of the results. This is supported by previous evidence of a false positive case of sebuthylazine residues (Schürmann, Dvorak, Crüzer, Butcher, & Kaufmann, 2009), false positive case of nitroimidazole residue (Kaufmann, Butcher, Maden, Walker, & Widmer, 2010) and a false negative case (Gallart-Ayala, Nuñez, Moyano, Galceran, & Martins, 2011) of benzophenone, which highlight the potential utility of high resolution mass spectrometry (HRMS) in food safety testing. The false positive case of sebuthylazine was due to an isobaric and endogenous compound (nepellitorine) in tarragon matrix, which caused significant variation in the monitored ion ratio, and then resolved using LC-TOF measurements. The false negative case of benzophenone was due to an isobaric interfering compound, harman, which caused a deviation in the ion ratio criterion beyond the tolerance range, leading to interpretation as a negative result. This interference was then resolved using an Orbitrap (Exactive) instrument and found to be a false negative.

Ion ratio criteria leading to false negative results have been studied extensively (Kaufmann, Butcher, Maden, Widmer, Giles and Uría, 2009; Mol, Zomer, & de Koning, 2012) and a broader ion ratio tolerance has been proposed (Mol et al., 2012). EU Decision 2002/657/EC (2002) sets out the criteria for confirmation based on the concept of identification points (IPs). According to this Decision, four identification points are required to confirm the presence of prohibited substances: one point for a precursor ion and 1.5 points for each transition product in case of a low resolution mass spectrometer (e.g. triple quadrupole analyzer). In addition, at least one ion ratio between two ions must be monitored and the tolerance criteria for ion ratio must be met, depending on the relative ion intensities.

Commission Decision 2002/657/EC has specific criteria for the use of HRMS, regarding identification points when monitoring specific ions, but it lacks information on mass measurement accuracy criteria, which makes this document inadequate for obtaining conclusive results. Some authors have discussed to update the criteria for using HRMS instrumentation (Nielen, van Engelen, Zuiderent, & Ramaker, 2007) and a set of guidelines has been proposed (Vanhaecke, Gowik, Bizec, Ginkel, Bichon, Blokland, et al., 2011). The Codex Alimentarius Commission is drafting an updated guidelines document (2012) on the performance characteristics for multiresidue analytical methods, where mass measurement accuracy when using HRMS instrumentation must be lower than 5 ppm.

Ronidazole belongs to the nitroimidazole family of antibiotics. Its use in food-producing animals is prohibited in the EU by three regulations, 3426/93/EC, 1798/95/EC and 613/98/EC (Eric, 2010). Several methods

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http://dx.doi.org/10.1016/j.meatsci.2014.02.004
0309-1740/

based on liquid chromatography mass spectrometry for analyzing ronidazole residues in muscle samples have been published and some selected publications in the last five years are reported here: (Granja, Nino, Reche, Giannotti, de Lima, Wanschel, et al., 2013; Tölgyesi, Sharma, Fekete, Fekete, Simon and Farkas, 2012; Xia, Li, Zhang, Ding, Jiang, Li, et al., 2008; Xia, Wang, Wang, Li, Zhong, Li, et al., 2013; Zeleny, Harbeck, & Schimmel, 2009; Zelníčková & Rejtharová, 2013). In this study, a suspected non compliant result in the analysis of ronidazole (Fig. 1) in muscle of different species using LC–MS/MS was found to be false positive using LC–HRMS (Q Exactive) measurements. Fig. 1 shows the structure of ronidazole (201 m/z) and its product ion (140 m/z).

2. Experimental

2.1. Chemicals and reagents

Standards of ronidazole and ronidazole d3 were obtained from Sigma-Aldrich (Seelze, Germany). Double deionized water (Milli Q, Millipore, Molsheim, France) of 18.2 $M\Omega cm^{-1}$ was used. All other reagents were of analytical grade.

Stock standard solutions of ronidazole and ronidazole d3 were prepared in acetonitrile:water (3:7) at a concentration of 1000 mg L^{-1} and stored for up to 1 year at −20 °C. The deuterated Internal standard was diluted to 2 and 0.2 mg L^{-1} using a mixture of acetonitrile:water (3:7). The working solutions of standards at a concentration of 0.2 and 0.05 mg L^{-1} were prepared in acetonitrile:water (3:7). Working solutions were stored for up to 3 months at −20 °C. Strata Silica SPE cartridges (500 mg × 6 ml) from Phenomenex (Torrance, CA, USA) were used. Whatman Mini-UniPrep PVDF filters with polypropylene housing (0.2 μm) from GE Healthcare UK Ltd., (Buckinghamshire, UK) were used to filter the samples.

2.2. Instrumentation

A Thermo Accela ultra high performance liquid chromatography (UHPLC) system coupled with a Maylab Switch column manager and a quadrupole–orbitrap hybrid (Q Exactive) mass spectrometer (Thermo, Bremen, Germany) were used. The chromatographic system was coupled to the MS with Heated Electrospray Ionization Source II (HESI II). HESI II conditions were: spray voltage, 3.5 kV; sheath gas flow rate (N_2), 35 units; capillary temperature, 300 ° C; S lens RF level, 50; heater temperature, 350 ° C. Nitrogen obtained from a nitrogen generator Zefiro (Clan Tecnologica S.L., Sevilla, Spain) was employed as both collision and damping gas.

Mass calibration for Orbitrap was performed every three days to ensure a working mass accuracy lower than or equal to 5 ppm. Pierce LTQ Velos ESI Positive ion and Pierce LTQ Velos ESI Negative ion calibration solutions from Thermo Fisher Scientific (Rockford, IL, USA) were used for external calibration of the mass spectrometer.

Xcalibur 2.2 software from Thermo Fisher Scientific (MA, USA) was used for instrument control and data processing. Q Exactive 2.0 SP 2 (tune application) from Thermo Fisher Scientific was used to control the mass spectrometer.

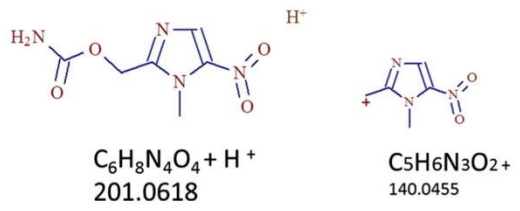

Fig. 1. The structure of ronidazole ($M + H^+$) ion and its monitored product ion.

A Waters Acquity UHPLC system (Manchester, UK) coupled to a Quattro Premier triple–quadrupole mass spectrometer from Micromass (Manchester, UK) equipped with an electrospray ionization source was used. The electrospray ionization source was operated in positive mode under the following conditions: capillary voltage, 3.5 kV; source block and desolvation temperatures, 120 ºC and 400 ºC, respectively; desolvation and nebulizer gas (N_2) flow rates, 650 lh^{-1} and 50 lh^{-1}, respectively; argon pressure in the collision cell, 4×10^{-3} mbar. Instrument control and data processing were carried out using MassLynx 4.1 software.

A Hettich refrigerated centrifuge (Tuttlingen, Germany) was used.

2.3. Muscle sample preparation for ronidazole analysis

A 3 g sample of minced porcine muscle (longissimus dorsi tissue) was added to a 50 ml centrifuge tube, spiked with 100 μl of 0.2 mg/l internal standard solution and 6 ml of acetonitrile (ACN) was added. For method matrix matched standards (matrix fortified standards), blank samples were spiked with specific volumes of the working standard solution up to concentrations of 1 to 50 μg/kg and then extracted. Samples were homogenized by manually shaking and vortexing for 15 s, ultrasonicated for 5 min, and centrifuged at 3500 rpm for 10 min. The supernatant was loaded on a silica SPE cartridge (500 mg) previously conditioned with 5 ml of ACN and the eluate was collected in a 15 ml glass tube. The cartridge was washed with 2 ml of ACN and dried under vacuum for 2 min. The eluate was then dried in a nitrogen evaporator at 40 ºC and reconstituted with 400 μl of water:acetonitrile (1:1) solution. The solution was vortexed, ultrasonicated, filtered with a 0.2 μm filter sample vials and volumes of 2 μl and 10 μl were injected into the LC–HRMS and LC–MS/MS, respectively.

2.4. Liquid Chromatography/mass spectrometry

The parameters of the multiple reaction monitoring method for the triple quadrupole mass spectrometer and the method parameters of Q Exactive are provided in Tables 1 and 2, respectively. A Kinetex 2.1 × 100 mm (1.7 μm) column from Phenomenex (Torrance, CA, USA) was used in both techniques. Mobile phase consisted of a mixture of 0.1 % aqueous formic acid solution (A) and acetonitrile (B). The gradient program (t, %B) used was: (0,0); (6,25); (8,0); and (10,0).

3. Results

In the laboratory of Barcelona Public Health Agency (ASPB), non compliant results for ronidazole in muscle samples had been obtained repeatedly even though it had been reported that ronidazole was not used for either growth promotion or treatment purposes. To assess the possibility that these were false positive results, these samples were re-analyzed using the HRMS instrument in order to clarify their status. Fig. 2 shows the results from the triple quadrupole analyzer (QqQ), where retention time matches the standard (within ±2.5% tolerance), two product ion peaks from the multiple reaction monitoring MRM transitions are present, the ion ratio between the two product ions in the sample does not vary markedly from the ion ratios in the standard (within ±25% tolerance) and the concentration is within the

Table 1
MRM transitions and collision energy in LC–MS/MS.

MRM	Collision energy (eV)
Ronidazole	
201.0 > 140.0	12
201.0 > 55.0	20
Ronidazole d3	
204.1 > 143.0	12

Table 2
Monoisotopic masses and collision energies in LC–HRMS.

	Chemical formula	Precursor ion				Product ion				
		Adduct	Exact mass	Accurate mass	Error (ppm)	Chemical formula	Exact mass	Accurate mass	Error (ppm)	NCE (%)
Ronidazole	$C_6H_8N_4O_4$	$[M + H]^+$	201.0618	201.0617	0.5	$C_5H_6N_3O_2^+$	140.0455	140.0455	0	35
Ronidazole d3	$C_6D_3H_5N_4O_4$	$[M + H]^+$	204.0807	204.0806	0.5					

calibration curve, thereby complying with the Decision 2002/657/EC criteria for reporting as a positive or non-compliant result.

These samples were then analyzed using the LC–HRMS in Full MS/All ion Fragmentation (AIF) mode at a resolving power of 70000 for both Full MS and AIF experiments. Fig. 3 (left) shows the extracted ion chromatograms (XIC) for the precursor ion (201.0618 m/z) and the product ion (140.0455 m/z), extracted using a mass window of 2000 ppm (~0.8 Da), simulating the QqQ mass resolution window, where precursor and products ion peaks were detected. No chromatographic peaks were seen while extracting with a mass window of 2 ppm (0.008 Da) in Fig. 3B and C (right), whereas a peak was observed for the spiked internal standard in Fig. 3A (right), confirming that the suspect compound is not ronidazole.

Concerning the product ion 55 m/z, although it is commonly followed in QqQ (Cronly, Behan, Foley, Malone, & Regan, 2009; Mahugo-Santana, Sosa-Ferrera, Torres-Padrón, & Santana-Rodríguez, 2010; Tamošiūnas & Padarauskas, 2009), the possible structure of the product was not identified. The XIC (55 m/z with 1 Da extraction window) of ronidazole standard injection, which simulates the resolution of triple quadrupole, did not extract any chromatographic peak. While inspecting at the mass spectrum of ronidazole chromatographic peak from the standard injection, an abundant ion, 55.0548 was found. The XIC of 55.0548 m/z at 5 ppm window showed the presence of ion throughout the chromatogram at intensity 10^6, Also, it was present in all the injections (samples, blanks and standards). So, it was decided not to follow m/z 55 because it is not a characteristic fragment ion in the HRMS method.

Fig. 4 shows the MS spectrum and MS/MS spectrum at the retention time (4.32 min) of ronidazole, where the mass peaks of the interferences can be seen. We queried the Chemspider database (Little, Williams, Pshenichnov, & Tkachenko, 2012) (mass tolerance 2 ppm) for information on the interfering peak masses (201.1117 m/z and 201.1229 m/z), but these did not appear to correspond to known contaminants. These recurrent interferences have been observed in 14

	Relative retention time (201.0>140.0/ 204.1>143.0)	Ion ratio (201.0>140.0 / 201.0>55.0)
Suspected Sample	1.002	4.635
Matrix matched standard	1.004	4.456

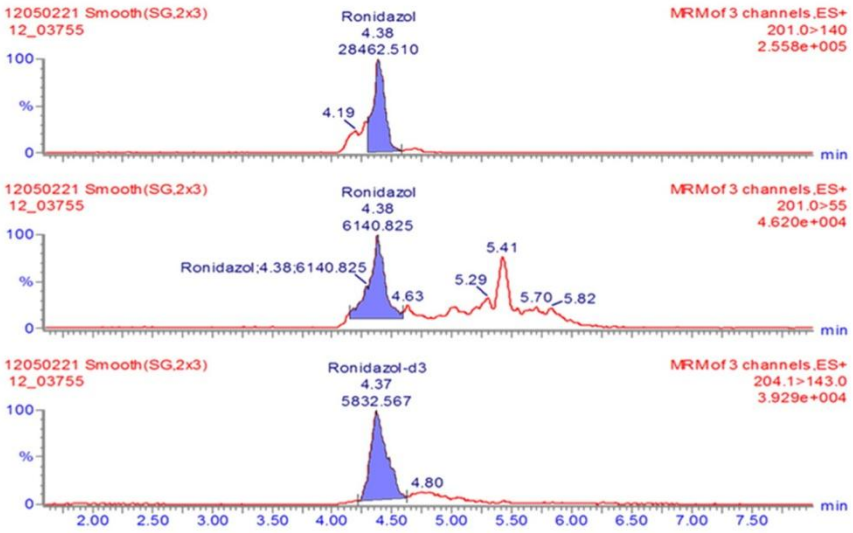

Fig. 2. Results of a suspicious positive sample for ronidazole from LC–MS/MS system.

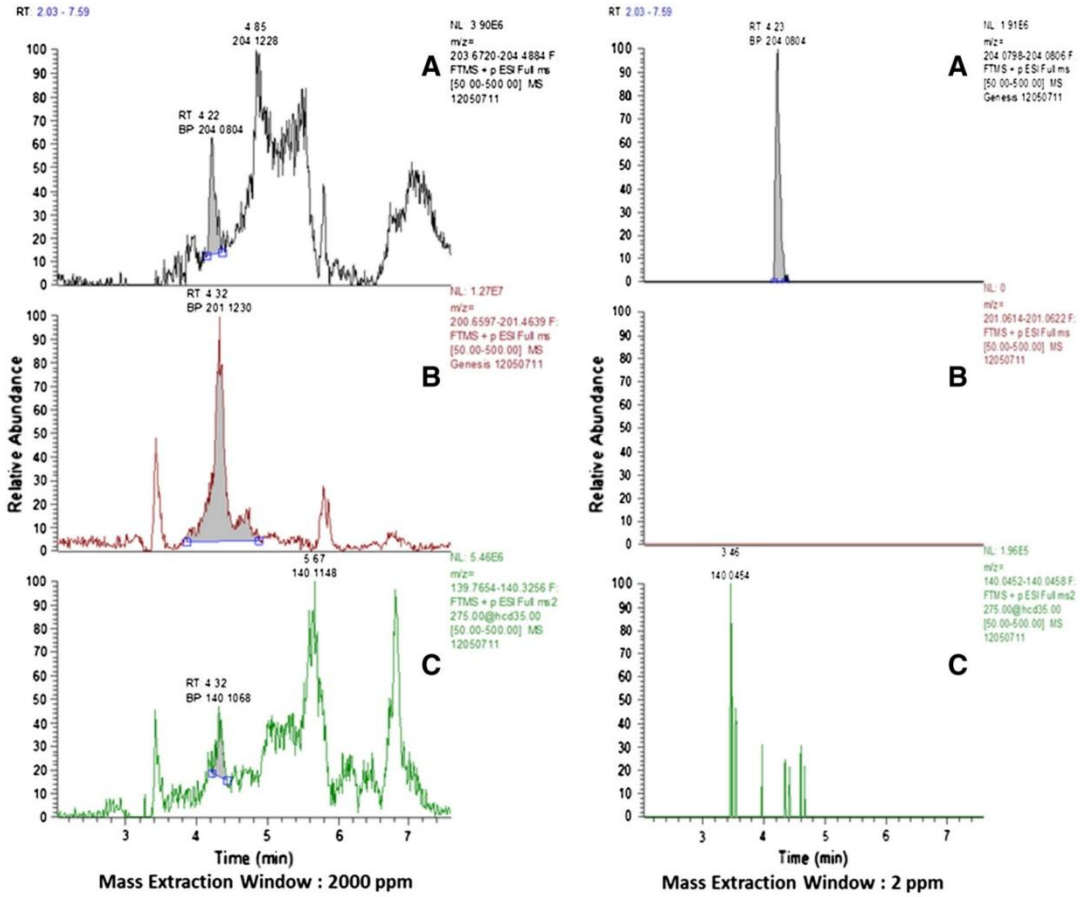

Fig. 3. Left: Extracted ion chromatograms (XIC) using a mass window of 2000 ppm simulating the resolution of a quadrupole analyzer. Right: XIC extracted with a mass window of 2 ppm. XIC of a ronidazole-d3 ionternal standard (A). XIC of ronidazole precursor ion (B) and product ion (C) of the suspicious sample.

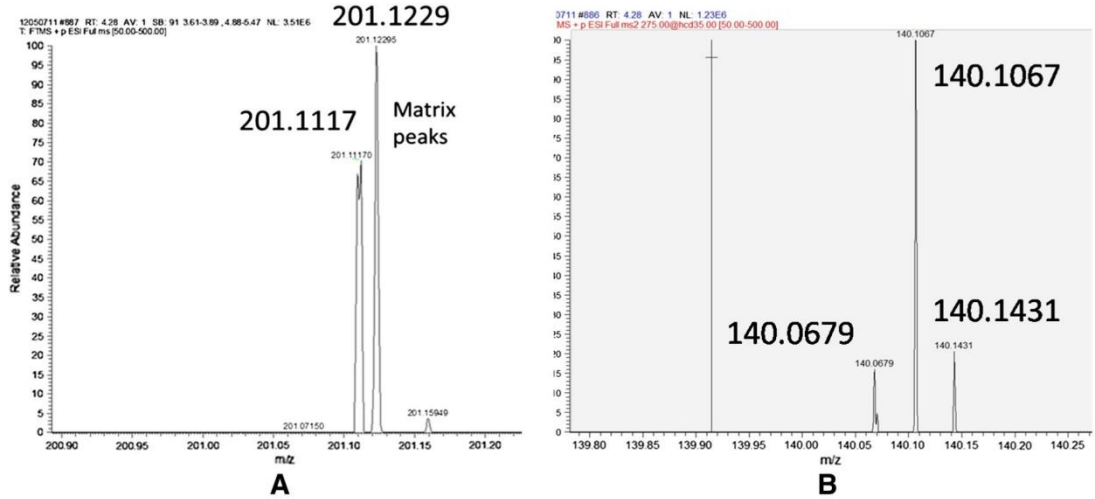

Fig. 4. Full MS spectrum at the retention time of ronidazole, showing the presence of isobaric matrix interferences (201.1117 m/z and 201.1229 m/z), where the exact mass of ronidazole is 201.0618 (A). MS/MS spectrum at the retention time of ronidazole showing the presence of isobaric interferences (140.0679, 140.1067 and 140.1431), where the exact mass of the product ion is 140.0455.

of 296 samples in the 6 months prior to this study. Muscle samples of pig, cow, sheep, horse, rabbit, turkey and poultry are regularly analyzed and the isobaric interferences are mostly found in pig, cow and rabbit samples.

4. Discussion

Reliable confirmation of the presence of chemical substances in food samples using mass measurements involves confirmation of their molecular weight, elemental composition and the structure of the compound. In food testing laboratories, triple quadrupole analyzers are still workhorses for confirmatory residue analysis and the use of HRMS is still scarce. The lack of resolution with triple quadrupole analyzers and limitations in confirmation criteria of Decision 2002/657/EC are the cause of some false-positive (Schürmann et al., 2009) and false-negative cases (Gallart-Ayala et al., 2011; Kaufmann et al., 2009). The implementation of HRMS for confirmatory analysis requires establishing adequate confirmation criteria. Although, HRMS has an advantage of measuring masses at high accuracy and high resolution, relying only on precursor mass measurements may not be selective enough when complex matrices with isobaric interferences are analyzed. In this sense, an assessment study by (Stoev, Xuan, Peycheva, & Scigelova, 2012) showed that relying solely on a precursor ion in HRMS would require a resolution of above 1 million in order to achieve the selectivity of selected reaction monitoring (SRM) in a triple quadrupole instrument (unit mass resolution). On the other hand, a comparative study by (Kaufmann et al., 2010) between LC–QqQ and LC–HRMS selectivity, concluded that accurate mass of precursor ion measured at 50,000 FWHM show higher selectivity than low resolution LC–MS/MS transitions. Moreover the article reports a false positive case with a dimetridazole residue analysis in honey with LC–QqQ, which was resolved with LC–HRMS.

In the confirmation of the identity of a chemical substance, if only retention time and accurate mass of precursor ion are monitored, it can produce false positive results when isobaric compounds with similar structure are present in the sample. When an accurate mass of a product ion is also monitored in addition, it verifies the substructure of the chemical compound and thereby it is more unlikely to have a false positive. Further, if ion ratio between two ions is monitored with tolerance levels based on scientific evidence, then selectivity would increase.

In order to show the selectivity of m/z measurements, an in-silico fragmentation search was carried out in MetFrag (Wolf, Schmidt, Muller-Hannemann, & Neumann, 2010). When accurate mass of ronidazole, 201.0618 m/z ($M + H^+$), was searched in Chemspider with 5 ppm mass tolerance, 92 compounds were found to match. When the accurate mass of the product ion 140.0455 m/z at 5 ppm tolerance was matched with in-silico fragments of the 92 compounds, only 22 compounds matched, showing increased selectivity. Out of these 22 hits, 21 compounds have the same molecular formula. When searched with low resolution m/z of ronidazole–201.06 ($M + H^+$) in Chemspider with 2000 ppm mass window, it gave 18,336 hits which is very high compared to 92 hits obtained with accurate mass search. Moreover, when product ion 140.01 m/z at 2000 ppm mass extraction window was matched with in-silico fragments, the list of matching candidates reduced to nearly half (2236 out of 5000 processed hits). More the matching compound hits for m/z measurements, less the selectivity of mass traces. According to the above study with Metfrag, selectivity increases in the following order: one low resolution transition product ion < one high resolution (HR)/accurate mass (AM) precursor ion < one HR/AM precursor & one HR/AM product ion. Moreover, if an ion ratio with appropriate tolerance is used as a criterion, the selectivity would increase. The results from this study questions the assignation of 1.5 IPs to a low resolution (LR) transition product and 2.5 IPs to a HR transition product. The IP for HR ion is approximately two times more than the IP for LR ion, whereas selectivity of HR ion is much higher than twice that of LR ion. Moreover, as the recent HRMS instruments have the capability to acquire accurate mass of fragment ions, it would be prudent to monitor fragments to attain the highest level of selectivitypossible. In order to use the HRMS measurements for regulatory purposes, Codex guidelines document (2012) specifies mass measurement accuracy criteria to be lower than 5 ppm, which was not specified by Decision 2002/657/EC.

In this article, a false-positive case of the presence of ronidazole, meeting all criteria of Decision 2002/657/EC, during routine use of a triple quadrupole analyzer is presented. When analyzed at high resolving power (70000 FWHM) using a HRMS instrument, this false-positive result was found to be due to isobaric matrix interferences. This case clearly shows the utility of the HRMS instrument in food safety laboratories in order to avoid false-positive results arising from matrix interferences. In case of using LC–MS/MS, as false positive cases are reported increasingly in the literature, it would be appropriate to monitor a third transition product ion and the corresponding second ion ratio to increase the selectivity. As an alternative to using LC–HRMS to resolve isobaric interferences, sample preparation and chromatographic conditions (stationary phase, pH, organic modifier and temperature) can be optimized to separate the interference from the analyte and increase the selectivity of the method.

Moreover criteria were established in the laboratory to use HRMS for confirmatory analysis and for the past six months our laboratory has been applying this method routinely using the LC HRMS. The criteria established are, retention time tolerance of $\pm$ 1%, at least one product ion at high resolving power (>20,000 FWHM), a minimum resolving power of 70,000 FWHM for precursor ions, mass measurement accuracy of less than or equal to 5 ppm, and monitoring of at least one ion ratio. Calibrating the Orbitrap once every 3 days is adequate to ensure mass accuracy less than 5 ppm.

Acknowledgments

P. Kumar thanks the Generalitat de Catalunya for an FI grant (Grant Number 2012 FI-B2 00217).

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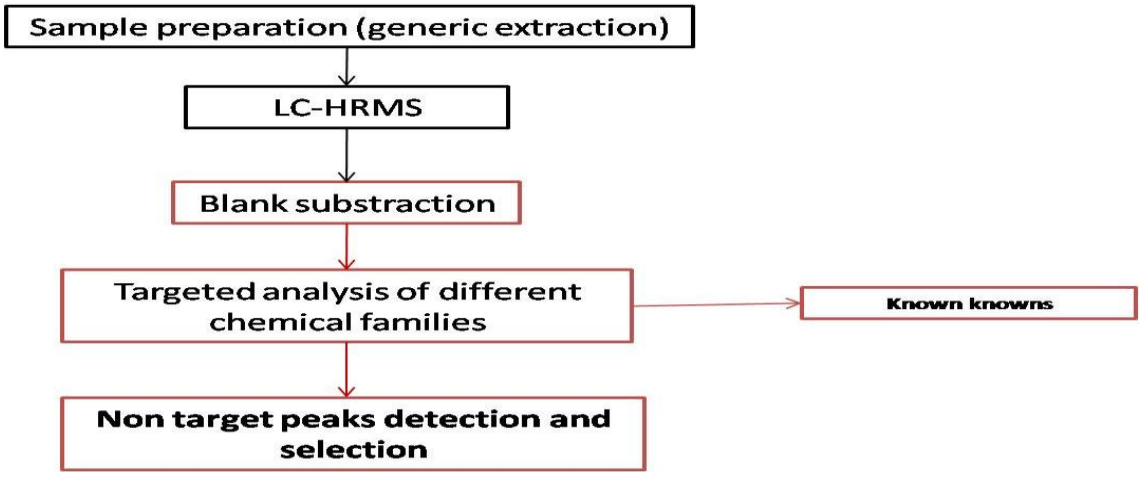
8. Non targeted analysis based on database approach with quadrupole-Orbitrap mass spectrometry data

8.1. Introduction

In food and environmental testing field, testing methods are developed to analyze or look for contaminants belonging to one or few families (targeted analysis) which the control authorities demand for. However, there is a high probability for the presence of other contaminants belonging to different chemical families such as pesticides, food contract materials, natural toxins, industrial contaminants etc, which can be introduced in any part of the food chain from farm to table. This inherently demands the change in testing methods from providing binary (residue present – non compliant /not present - compliant) results to comprehensive test results (safe/unsafe sample). In an analytical perspective, this would need development of non targeted analysis strategies.

The Orbitrap based instruments have evolved rapidly in recent years and the quadrupole – Orbitrap hybrid instrument (Q Exactive) provide good performance characteristics such as high resolving power, accurate mass measurements, full scan capability and the presence of quadrupole to isolate precursors for analyzing complex sample matrices in residue analysis.

The comprehensive analysis of a test sample involve a generic sample extraction step, chromatographic separation, high resolution mass spectrometric detection and followed by targeted and non targeted analysis. A systematic workflow adapted from literature [88] [89] is given in figure 8.1 .



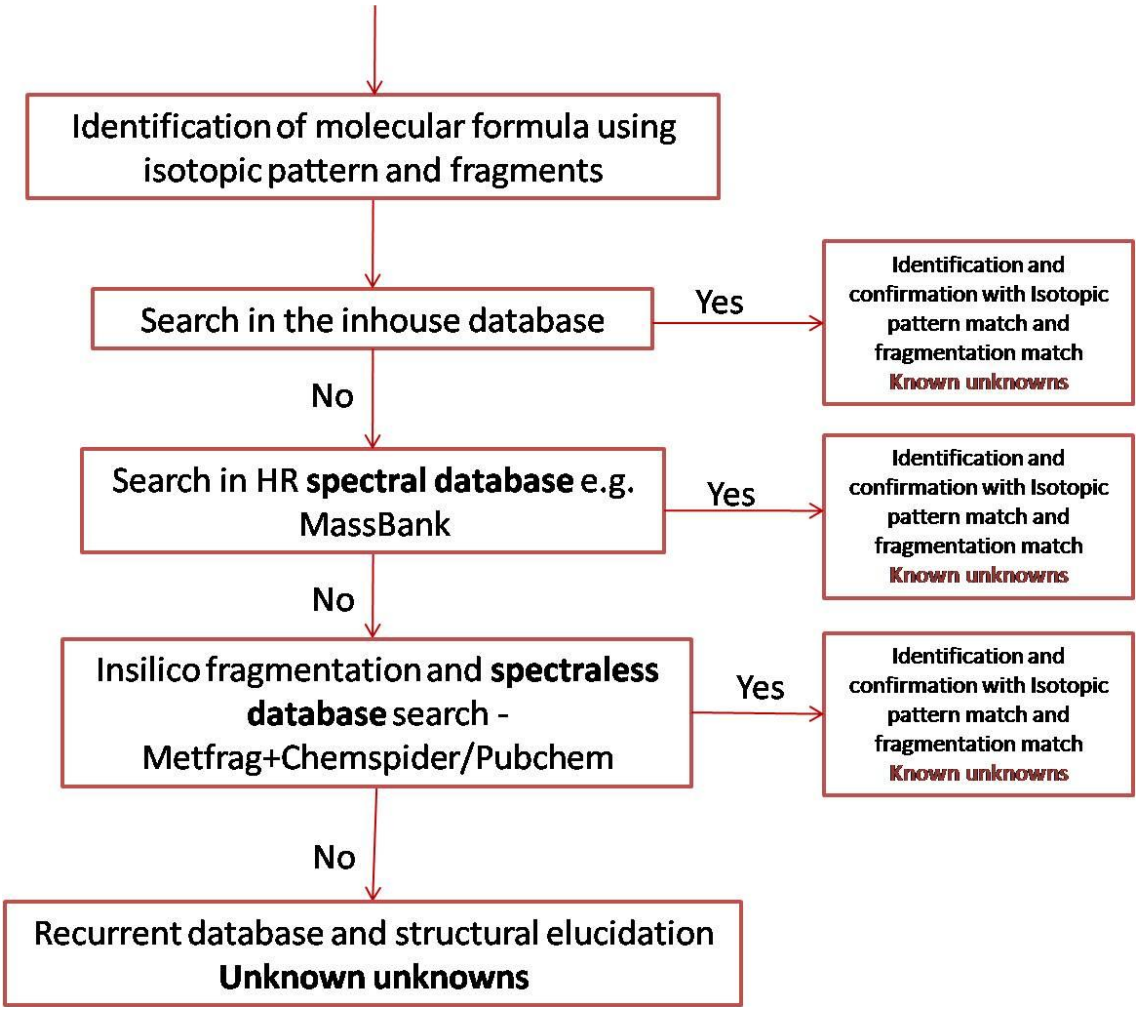


Figure 8.1. Systematic workflow of targeted and non targeted analysis

Targeted analysis is for substances for which method is validated with reference standards. Suspect screening approach is for substances with some previous information. Non- targeted analysis focuses on substances without any previous information. It has to be mentioned that, non targeted screening based on database approach depends largely on spectral libraries and adequate data analysis tools [133].

Although different authors use various terms for non targeted analysis, the classification based on workflow leads to two categories – targeted and non targeted analysis. Classification of a substance after the compound is identified can be categorized into - known knowns, known unknowns and unknown unknowns. Known knowns are the targeted compounds. Known unknowns are the compounds unexpected by analyst but indentified in comprehensive libraries. Unknown unknowns are the compounds

unexpected by analyst and not identified in comprehensive libraries. In a control lab perspective, it would be practical to create a recurrent database for unknown unknowns and try to elucidate the structure for the ones with high frequency occurrence in the sample.

An Orbitrap data obtained using any quadrupole modes as described in chapter 4, can provide accurate mass, adduct, isotopic pattern and precursor isolated MS/MS fragmented at different collision energies for each ionized chemical substance. The puzzle in non targeted analysis is to identify the chemical substance from these features (Figure 8.2).

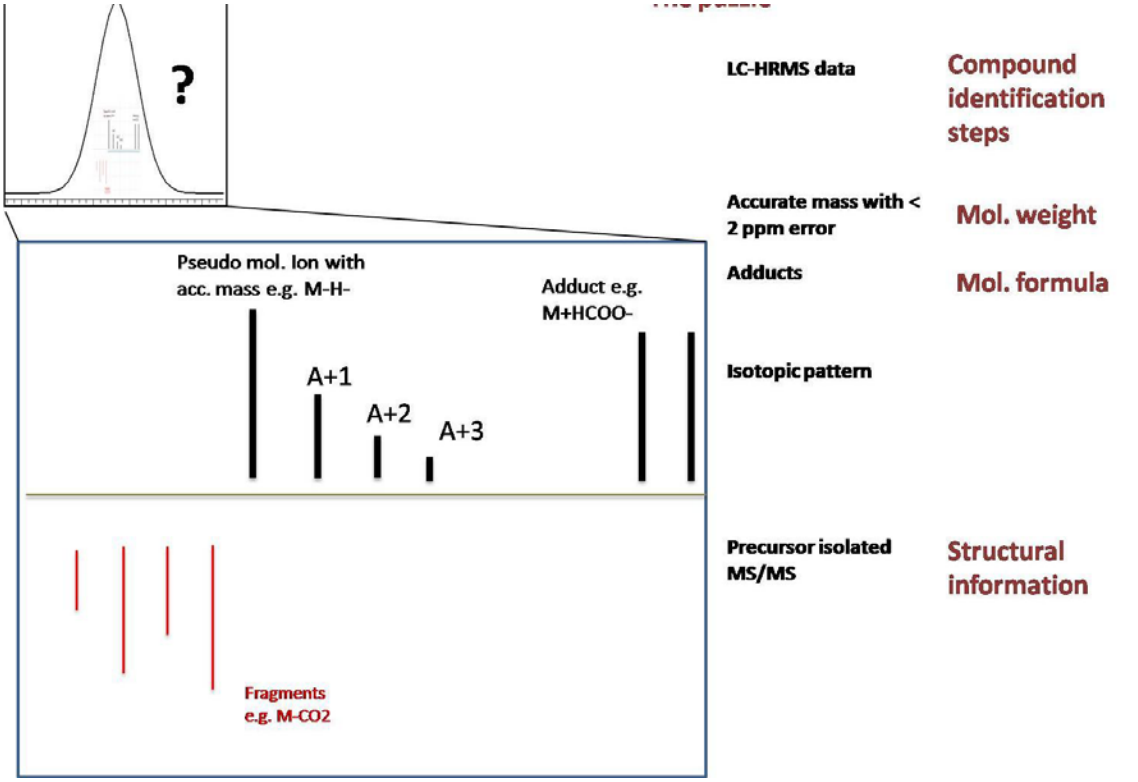


Figure. 8.2. Representation of LC-HRMS data and compound identification steps in non targeted analysis

This involves the use of various data analysis tools. In this scenario, the objective of this work was to develop a data analysis workflow for non targeted analysis for negative ionization data, and apply it to a sediment core sample. This work was carried out during a research stay at Environmental Chemistry group of EAWAG (Swiss Federal

Research Institute). The results from this work are included in a scientific article which is in preparation [134].

8.2. Materials and methods

The sample preparation and chromatographic method was developed by Aurea Chiaia-Hernandez et al [135]. In brief sample preparation involves pressurized liquid extraction followed by a dispersive solid phase extraction step.

The separation of all analytes was performed on a 2.1 × 10 mm C18 security guard cartridge connected to a 2.1 × 50 mm × 3.5 µm particle size X-bridge C18 column (Waters Corp., Milford, MA) at 35 °C. Q Exactive with acquisition mode - Full MS ddMS/MS using the inclusion list was used. A resolving power of 70,000 FWHM and an accuracy < ±5ppm was ensured by calibration. A precursor isolation width of ± 4 Da was used. Flucofuron and hexachlorophene reference standards (purity ≥ 97%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). All reagents used were of analytical grade.

Tools

X Calibur 2.2 software from Thermo Scientific (MA, USA) was used to control the instrument and data acquisition. Q Exactive 2.0 SP2 (tune application) from Thermo Scientific (MA, USA) was used to control the mass spectrometer.

Envimass, an software developed in house by EAWAG was used. It is a tool for automated screening of targeted compounds and internal standards from raw data and group non targeted data. Peak detection software, Formulator (Thermo) was used to generate input files for Envimass. The Envimass is implemented in Excel/visual Basic and also utilize RExcel for using Rstatistical environment [136].

Molgen MSMS, a command line program that use MS and MS/MS data to predict molecular formulas for non targeted candidates was used. The program use isotopic pattern match of MS data, assign subformula to MS/MS peaks to score molecular formula candidates. The output includes mass deviation (ppm), MS match value (MS MV), MS/MS match value (MS/MS MV) and combined match value (Comb MV), a direct multiplication of MS and MS/MS MV [137, 138]. An example command line settings used in this work is as follows:

Syntax : Molgenmsms ms=317_0823_ms.txt msms=317_0823_msms.txt ion=-H cha=-1 exist ppm=5 acc=10 el=CHNOPSClBr oei out analyze loss sort=combmv m=317.0815

Explanation : ms – filename of MS data, msms – filename of MSMS data, ion – type of ion, cha – charge of ion, ppm – accuracy of measurement, acc – allowed deviation for acceptance of MS/MS peaks in ppm, el – used chemical elements, oei – allow odd electron ions for explaining MS/MS peaks, analyze – write explanations for MS/MS peaks, loss – output neutral losses, sort – sort generated formulas according to combmv, m – mass of MS base peak.

Metfrag, an open source application with web interface for identifying product ions of small organic compounds, was used. It use the MS/MS peak list and calculated exact mass of the compound as input and search databases such as Pubchem and chemspider for matching candidate compounds. Each candidate is then fragmented insilico and every possible structure is generated and ranked based on measured and Insilco fragment match [85].

Chemspider, spectraless database of more than 26 million unique molecules; It was acquired by Royal society of chemistry in 2009. **Pubchem** is another spectraless database containing more than 3 million entries of compounds. Pubchem is maintained by National Institute of Health (NIH), United States. Accurate mass data can be used to query the database to identify the compounds with MetFrag interface [82, 83].

ToxID 2.0, an automated compound screening tool, was used. It uses an excel input file with compound name, retention time with tolerance, ionization mechanism, molecular formula. It creates output as report files in pdf and excel with chromatographic peak of each compound and found parameters. The program settings also allow MS/MS ions.

RT correlation using log K_{ow} was used at ± 4 mins interval. Log K_{ow} represent a good model for retention of neutral compounds on C18 columns [139]. A correlation between log K_{ow} and experimental retention time of 164 reference standards were calculated and used to predict the retention time for non targeted compounds. The correlation is as follows:

$$\text{Predicted RT} = 1.65 * \log K_{ow} + 4.36$$

8.3. Results and discussion

The identification of non targeted peaks (from mass to compound identification) involve determining molecular weight, molecular formula, structural information of the ion from instrumental measurement features such as predicted/actual RT, accurate mass, adduct, isotopic pattern and precursor isolated MS/MS at different NCEs. In this work, only negative raw data was used for data analysis, as it is less explored.

Using Envimass, blank data was subtracted, instrumental noise and chemical noise peaks were removed and recalibration of data was carried out with internal standards. The subtraction of noise, blank, target peaks gave 5435 non targeted peaks and 88 peaks out of them contained Cl,Br and Si. (Figure 8.3)

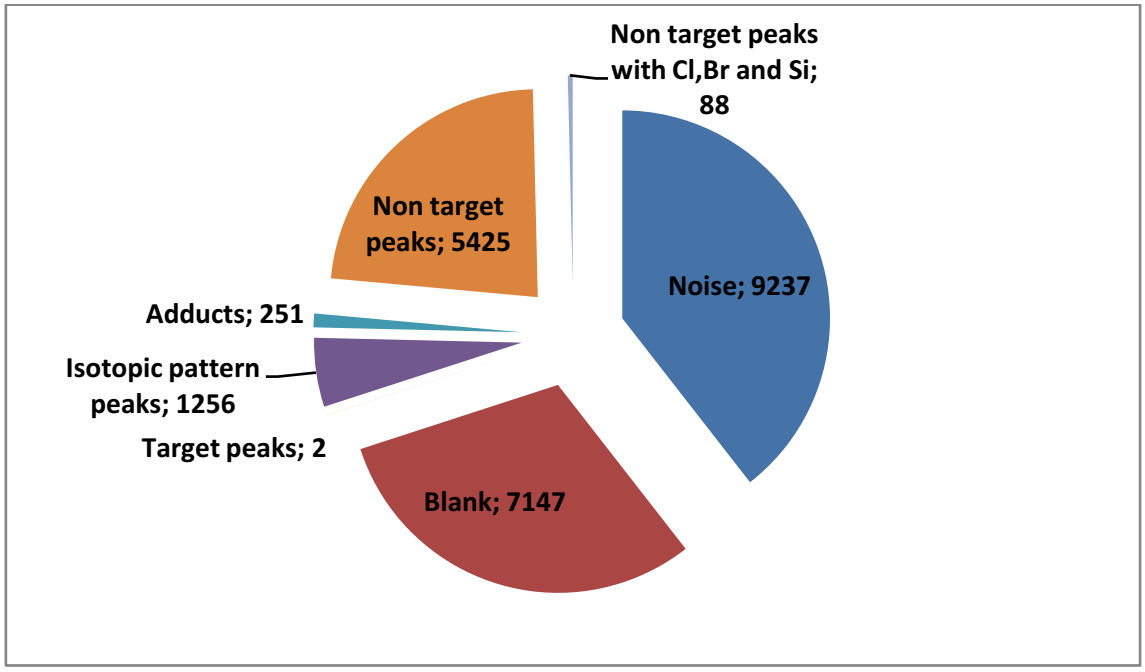


Figure 8.3. Peak identification of HRMS data in negative ionisation

Suspect Screening

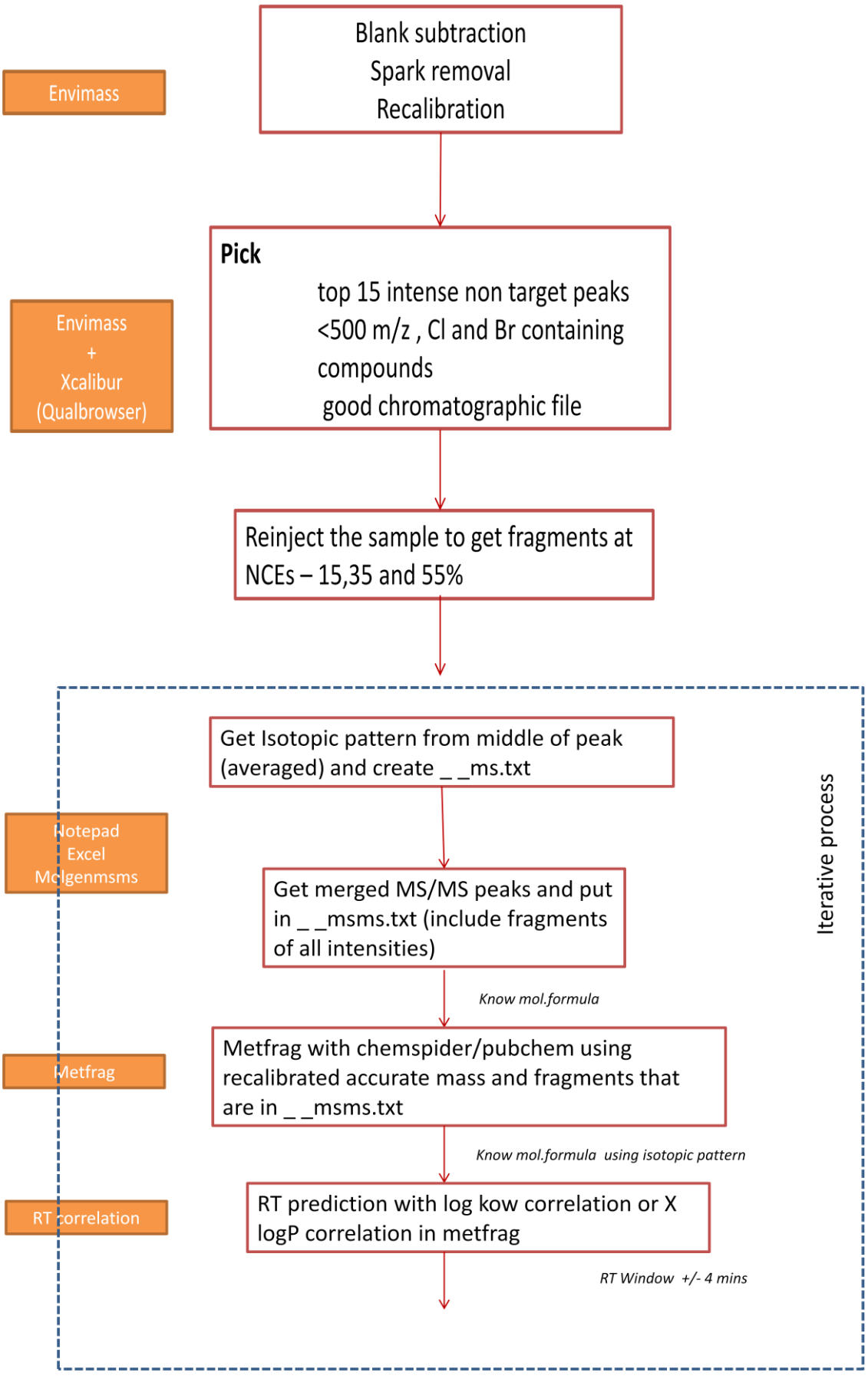
Suspect screening was carried out with ToxID and then iteratively each peak hit from ToxID was verified for retention time match and isotopic pattern match. The isotopic pattern match was carried out by visual check with XCalibur. The results are summarized in table 8.1. The table lists the compounds with retention time match and isotopic pattern match. The full confirmation of their identification would require matching MS/MS product ions and verification with reference standards.

Table 8.1. Results of suspect screening of sediment sample with negative ionization

Predicted RT					Actual RT	RT match	Isotopic Pattern Match
13.2	hexaflumuron	C16H8Cl2F6N2O3	-	460.99069	15.91	YES	YES OR NO
	3,5, 6-trichloro-2-pyridinol	C5H2Cl3N1O1	-	195.91312	9.64		YES
14.5	BROMCHLOROPHENE	C13H8Br2Cl2O2	-	422.81989	15.91	YES	YES
11.3	CLOROFENE	C13H11Cl1O1	-	217.04291	12.83	YES	YES
15.8	**HEXACHLOROPHENE**	**C13H6Cl6O2**	-	**402.84247**	**17.05**	**YES**	**YES**
11.7	KETOCONAZOLE	C26H28Cl2N4O4	-	529.14362	14.45	YES	YES
11.6	Soneclosan	C12H8Cl2O2	-	252.98343	13.53	YES	YES
12.5	**Triclocarban_2**	**C13H9Cl3N2O1**	-	**312.97131**	**14.27**	**YES**	**YES**
12.6	**Triclosan_2**	**C12H7Cl3O2**	-	**286.94424**	**14.56**	**YES**	**YES**
9.2	**Diuron**	**C9H10Cl2N2O1**	-	**231.01003**	**8.84**	**YES**	**YES**
10.3	Chlorophen	C6H1Cl5O1	-	262.84015	15.32	NO	YES

Non targeted screening

In this study, non targeted analysis based on database approach has been used. The workflow is an iterative process for each non targeted peak hit. The MS mass list and MS/MS merged ion list were used in Molgen msms and molecular formula were predicted. Although using Molgen msms is advantageous to predict molecular formulas from isotopic pattern and MS/MS than prediction molecular formula from only accurate mass. The isotopic pattern match (MS match value) is calculated between MS peak list and candidate molecular formulas. More the ions in MS peak list match, more the score. Because of this, noise peaks have to be removed before input into Molgen ms/ms. This is a drawback because, it is not easy to differentiate between an analyte mass peak and noise peak. If the score is calculated based on candidate molecular formula isotopic pattern match, this issue could be solved. In the proposed way, more the mass peaks of candidate molecular formula match with sample MS list, more the score. This would increase the reliability of MS match value score.


Envimass
Blank subtraction
Spark removal
Recalibration
Envimass
+
Xcalibur
(Qualbrowser)
Pick
top 15 intense non target peaks
<500 m/z , Cl and Br containing compounds
good chromatographic file
Reinject the sample to get fragments at NCEs – 15,35 and 55%
Notepad
Excel
Molgenmsms
Get Isotopic pattern from middle of peak (averaged) and create _ _ms.txt
Get merged MS/MS peaks and put in _ _msms.txt (include fragments of all intensities)
Know mol.formula
Metfrag
Metfrag with chemspider/pubchem using recalibrated accurate mass and fragments that are in _ _msms.txt
Know mol.formula using isotopic pattern
RT correlation
RT prediction with log kow correlation or X logP correlation in metfrag
RT Window +/- 4 mins
Iterative process

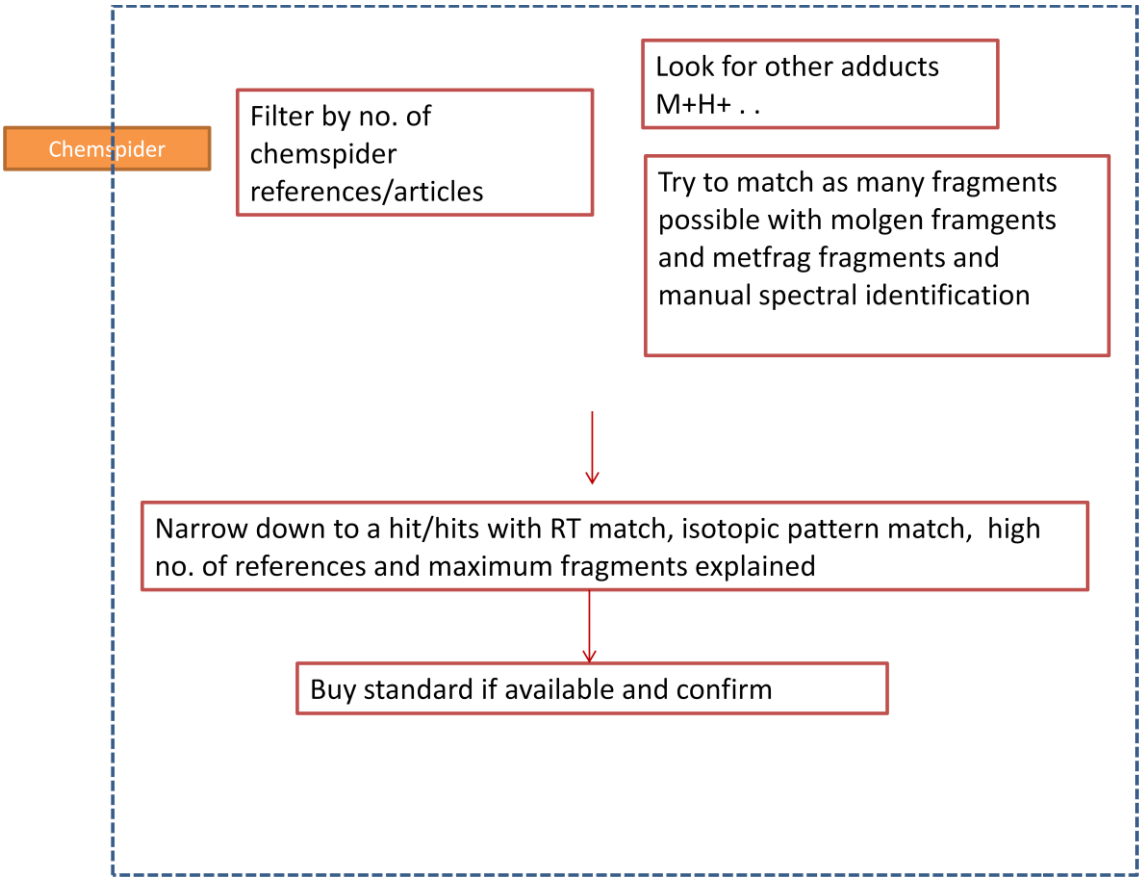


Figure 8.4. Step by step workflow developed for non targeted analysis

The accurate mass and MS/MS list of unknown peak is then searched in Chemspider and Pubchem in Metfrag interface at 2 ppm interval and all the candidates were fragmented insilico, match score with MS/MS list is calculated and the candidate list is sorted.

From the Metfrag candidate results, predicted RT was calculated using XlogP correlation. The shortlisted candidates were then filtered by no. of Chemspider reference, other clues – adducts in positive ionization, match as many fragments as possible.

The step by step workflow is shown in figure 8.4.

The list of non targeted candidates is then narrowed down using criteria such as RT match, isotopic pattern match, high number of references and maximum fragmentation

explained. The standards can be purchased if available to confirm their identity. Two such non targeted peaks confirmed with standards are flucofuron and hexachlorophene (table 8.2).

Table 8.2. Summary of results from data analysis tools of two unknown compounds

Molgenmsms mol.formulas without element restriction	1321	Too many
With el restriction	2	143
Metfrag hits with msms	18	298
Rank in metfrag	1	1
Chemspider, no. of references	909	22
Sort mol.formulas with no. of references (Rank)	1	1
	Hexachlorophene	**Flucofuron**
Use	Common disinfectant, in soaps and toothpast, as fungicide in agri	Mothproofing agent in textiles/wool

The chromatogram and mass spectrum of hexachlorophene is shown in the figure 8.5.

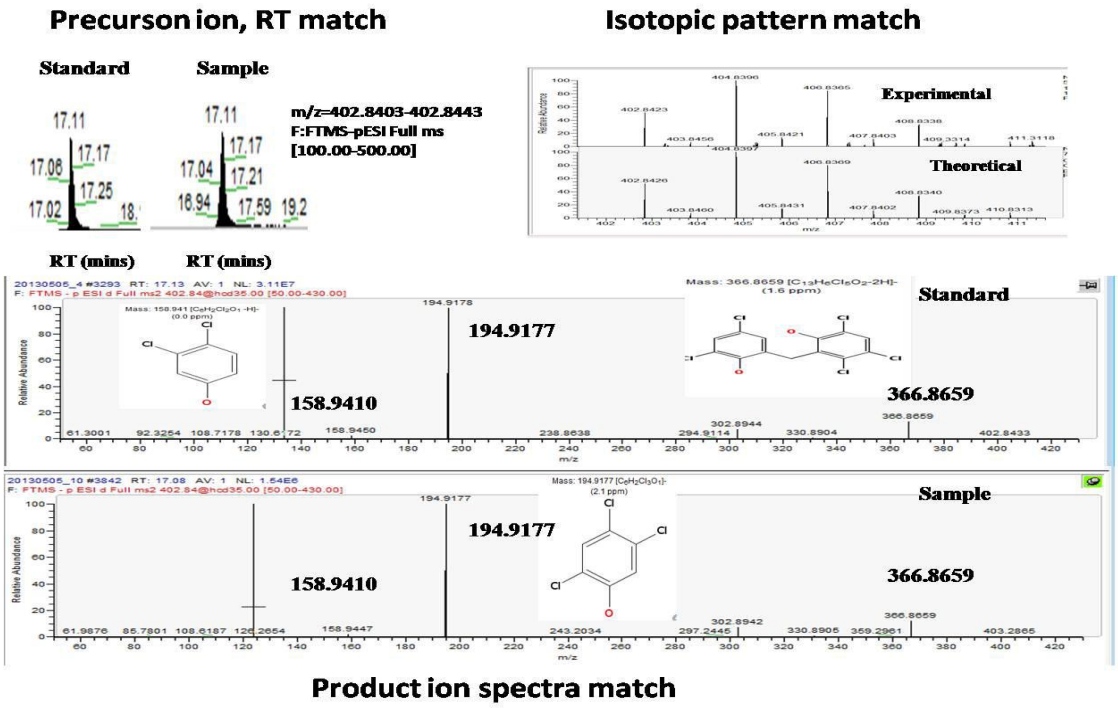


Figure 8.5: Retention time, product ion spectra, isotopic pattern match of hexachlorophene compound in standard and sample injections.

8.4. Conclusion

A systematic workflow for non targeted analysis with LC-MS/HRMS (Q Exactive) for Cl, Br containing non targeted peaks has been developed. Cl and Br containing compounds represent 19.2% of registered compounds in Chemspider. It can be further applied to other compounds. Two known unknown peaks have been identified and confirmed with standards as hexachlorophene and flucofuron in the studied sample.

Molgenmsms is advantageous to predict molecular formulas using fragments and assign molecular formulas of the products. However if isotopic pattern match score is calculated based on candidate list, it would improve the workflow dramatically to shortlist the possible candidates. Spectral libraries with fragment spectras are crucial for identifying non targeted. Currently spectral libraries with accurate mass data are scarce and are not exhaustive either. Users input to libraries such as Massbank and m/z Cloud could increase the ease of non targeted analysis. It has to be noted that non targeted analysis is still time and skill intensive. For routine analysis of samples, automated or semi automated workflows combined with extensive high resolution spectral libraries would be necessary.

IV. Conclusions & outlook

A simple method based on LC-UVfor analysing sulfonamides in various animal feeds has been developed.

Aminoglycosides is a difficult family of veterinary drugs having challenges to develop analytical methods due to its highly polar nature. Various HILIC phases for chromatographic retention of aminoglycosides have been systematically explored and methods with ZIC HILIC-MS/ MS has been developed to analyze aminoglycosides in honey and kidney samples. Further, a robust and simple chromatographic method based on amide HILIC-MS/HRMS has also been developed.

The potential of a hybrid quadrupole - Orbitrap hybrid instrument has been explored for food safety testing analysis. Different acquisition modes of the instrument have been thoroughly explored and a method to analyze hormones in urine has been developed.

A false positive result attained from a low resolution LC-MS/MS method was resolved with LC-MS/HRMS and the suspicion was found to be due to isobaric matrix interferences, demonstrating the selectivity of HRMS mass measurements. This also highlights the need for adequate confirmation criteria for test results.

A non targeted screening workflow using various data mining and analysis tools has been developed and applied to an environmental sample. Some suspect compounds and two unknown compounds have been identified.

In the last few decades, many countries/regions started creating centralized food safety systems and follow risk analysis framework fostered by WHO to undertake regulatory decisions regarding food safety based on science. This trend is followed by many other countries. Effectiveness of such a detective food safety control system depends on how fast a hazard is identified and controlled/removed from the food chain. Currently such system requires years to effectively control hazards in the food chain due to extensive science based risk assessment and risk management processes. The backbone of such system is the network of laboratories with pyramidal hierarchy (NRL, CRL, OCL) that provide evidence for policy managers by regular monitoring of hazards. The analytical method used by the laboratories should provide fast, accurate, affordable tests and consequently increase the sampling population to ensure solid evidence for policy managers. However, laboratories are often faced with challenges such as being updated with new technologies, ever increasing number of contaminants, lack of skilled personnel for rapid method development and tedious quality assurance protocols. Such crucial challenges could be addressed with industry-academy-research institute partnerships.

Moreover, advances in mass spectrometry especially in HRMS instrumentation provide interesting possibilities for food safety testing. Recent advances in mass spectrometry for microbial identification provide a accurate analytical tool for microbial testing, which is the major hazard in food safety. In contaminant analysis, hybrid HRMS instruments can minimize sample preparation procedures, comprehensively analyze the sample and provide test results as safe/unsafe sample rather than conventional yes/no results (targeted analysis). Wide scope and accurate testing methods can minimize personnel time providing the possibility to minimalize a food testing lab to an instrument and thereby minimalize the whole food safety system and the minimized uncertainty of test results improve efficiency of the system. Such transforming potential of HRMS technology in food testing field may address some important questions: Can I analyze a sample without reference standards? A non targeted analyte in an unregulated matrix can be found. How to proceed to regulatory decisions in such a case? Rapid (high throughput) and high performance methods are possible to be developed with HRMS instruments. Can it reduce the price of the analysis and provide affordable tests? Can OCLs include environmental analysis and clinical analysis testing and provide rich

data of presence of contaminants over the whole food and environmental chain for policy managers? Can pyramidal hierarchy in laboratories be minimized, the authority decentralized and responsibilities of OCLs increased? Can detective and preventive control be done at OCL level, when decentralized?

It has to be stressed that preventive control strategies like planting one´s own food plants and trees and avoiding the use of food hazards like pesticides and veterinary drugs could be more efficient way to ensure food safety than growing HRMS/population and also ensure sustainability.

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VI. Resumen en Castellano

Tal como afirma la Organización para la Agricultura y la Alimentación (FAO) de las Naciones Unidas en el documento "Directrices voluntarias en apoyo de la realización progresiva del derecho a una alimentación adecuada en el contexto de la seguridad alimentaria nacional", el derecho a la alimentación adecuada se ejerce cuando las personas tienen acceso a alimentos que,

- Proporcionan un valor nutricional suficiente y micronutrientes necesarios para que una persona pueda tener una vida sana y activa
- Están libres **de sustancias nocivas**
- Son aceptables para una cultura determinada,

Debido a la práctica de la agricultura intensiva e industrial, a la globalización y a la centralización de las cadenas de producción y de suministro de alimentos, el tipo de riesgos para la seguridad alimentaria fueron cambiando a lo largo del siglo XX. Anteriormente, los peligros más comunes de seguridad alimentaria eran los adulterantes. Hoy en día, una muestra de un producto alimentacio puede contener muchos otros productos químicos no deseados además de los adulterantes y esto ha provocado una transformación importante en los laboratorios de análisis para el control de los alimentos y en el enfoque de los propios métodos de análisis de adulterantes, residuos y contaminantes.

Un riesgo es un agente biológico, químico o físico, o propiedad de un alimento, con el potencial de causar un efecto adverso para la salud. Los peligros relacionados con la de seguridad alimentaria se pueden clasificar en tres categorías principales: químicos, microbiológicos y físicos. Los riesgos químicos pueden ser introducidos en la cadena alimentaria durante la producción, procesado, suministro, preparación y consumo.

Algunos episodios relacionados con crisis en la seguridad alimentaria en la Unión Europea focalizaron una gran atención por parte de los medios de comunicación y provocaron la pérdida de confianza de los consumidores. Con el fin de proteger la salud pública y garantizar la seguridad de los alimentos desde la granja hasta la mesa, muchos gobiernos en el mundo comenzaron a actualizar la legislación relacionada con la seguridad alimentaria y crearon organismos centralizados de seguridad alimentaria. En 2002, la Organización Mundial de la Salud (WHO), desarrolló una estrategia global

sobre la prevención de las enfermedades transmitidas por los alimentos, que actualmente se está implementando en muchos países. Esta estrategia comprende un enfoque holístico, y un análisis de riesgo basado en las políticas de seguridad alimentaria y la aplicación de las estrategias oportunas.

La función principal de las agencias o entidades reguladoras en este ámbito es la gestión de riesgos para la salud pública en los alimentos mediante un enfoque de análisis de riesgo (Figura 1). El análisis de riesgos consiste en la evaluación de riesgos, la gestión de riesgos y comunicación de riesgos.

Figura 1. Análisis de riesgo según el Codex Alimentarius

Un riesgo es una función de probabilidad de un efecto nocivo para la salud y de la gravedad de dicho efecto, como consecuencia de un peligro(s) en los alimentos.

Los medicamentos veterinarios son uno de los peligros químicos que plantean riesgos para la salud, tales como la resistencia a los antimicrobianos, alergías, toxicidad, etc. y su presencia en la cadena alimentaria está controlada por las autoridades de seguridad alimentaria. En la Tabla 1 se resumen los principales grupos de medicamentos veterinarios y sus usos.

Tabla 1. Resumen de las clases de medicamentos veterinarios y sus aplicaciones.

Tipo de medicamento	Aplicaciones, comentarios
Antihelmínticos	Se usan contra los gusanos intestinales, lombrices pulmonares, etc. Los grupos más utilizados son los benzimidazoles y las ivermectinas
Antibióticos	Se usan con finalidad terapéutica y también profiláctica
• Aminoglucósidos	Utilizados para tratar infecciones bacterianas Gram negativas. Los aminoglucósidos muestran nefrotoxicidad y ototoxicidad
• Beta – Lactamas	Activas contra bacterias Gram positivas y Gram negativas. Las penicilinas y las cefalosporinas son las β-lactamas más empleadas.
• Macrólidos	Activos ante bacterias Gram positivas – Utilizados para tratar enfermedades de las vías respiratorias.
• Péptidos	Activos frente a bacterias, virus y patógenos eucariotas. Se metabolizan más fácilmente que las moléculas pequeñas.
• Sulfonamidas y trimetoprim	Son bacteriostáticos utilizados frente a bacterias Gram positivas y negativas. El trimetoprim muestra un efecto sinérgico

	con las sulfonamidas. Muestran carcinogénesis.
• Tetraciclinas	Utilizadas frente a bacterias Gram positivas y negativas
• Quinolonas	Utilizadas frente a bacterias Gram positivas y negativas
• Cloramfenicol	Utilizado frente a bacterias Gram positivas y negativas
• Verde de malaquita	Utilizado para tratar ectoparásitos en acuacultura.
Coccidiostáticos- ionophores, nitroimidazoles y nitrofuranos	Utilizados para tratar y prevenir la coccidiosis.
Hormonas • Esteroides anabolizantes	Admistrados a los animales para aumentar la velocidad de crecimiento.
• Corticoesteroides	Utilizados como agentes antiinflamatorios.
• Tireostáticos	Inhiben el funcionamiento de la glándula tiroides y reducen la circulación de hormonas tiroideas. Esto provoca una mayor retención de agua por parte del animal, y en consecuencia un aumento de peso.
Beta-agonistas	Utilizados para tratar enfermedades respiratorias.

	Estos compuestos se acumulan en la retina de terneros, cerdos y pavos.
Tranquilizantes	Se administran a los animals antes de su transporte, para que no se produzca stress que disminuya la calidad de la carne.

En la UE la Directiva 96/23 CE establece las medidas de control de medicamentos veterinarios, plaguicidas y contaminantes en alimentos de origen animal. Este documento clasifica las sustancias no autorizadas y las sustancias con efecto anabolizante como el grupo A y los medicamentos veterinarios de uso autorizado y los contaminantes como grupo B.

Para controlar la presencia de sustancias químicas como los medicamentos veterinarios en los alimentos de origen animal, se han establecido los niveles máximos de residuo (LMR), que están basados en criterios científicos. Los LMR se obtienen como resultado de análisis de riesgos. El propósito de los LMR es proteger al consumidor, asegurándose de que los medicamentos veterinarios que se consumen en los alimentos de origen animal no sobrepasan la ingesta diaria admisible (ADI) de la sustancia. ADI se calcula a partir de un nivel sin efectos adversos observables (NOAEL), que luego se corrige con un factor de seguridad que tiene en cuenta las diferencias entre los animales y entre las personas.

$$ADI = NOAEL / \text{Factor de seguridad x masa de cuerpo}$$

Así, los medicamentos veterinarios de uso autorizado tienen establecidos valores de LMR, que para un mismo compuesto pueden variar dependiendo de la especie animal y del tipo de alimento o tejido.

De acuerdo con la Decisión de la Comisión 2002/657/CE de la UE, para las sustancias prohibidas, los métodos de análisis deben un límite técnica basada analítico llamado límite de funcionamiento mínimo exigido (MRPL) se debe utilizar. MRPL es la concentración mínima de analito en una muestra que ha ser detectado y confirmado. Se tiene que mencionar que, para muchas de tales sustancias, no se han establecido valores de MRPL.

La Decisión 2002/657/CE de la Comisión, indica los criterios que deben cumplir los métodos de análisis que se utilizan en el control oficial de residuos de medicamentos veterinarios en alimentos de origen animal. Asimismo proporciona criterios y procedimientos para la validación de métodos de análisis y pata la interpretación de los resultados analíticos de los laboratorios oficiales. Según este documento, los métodos se clasifican como métodos de cribado o métodos de confirmación. Los métodos de cribado deben proporcionar menos de un 5% de error de las muestras de falso conforme al nivel de interés. Las muestran que han sido identificadas como no conformes con el método de cribado deben ser comprobadas con un método de confirmación. Convencionalmente, los métodos de cribado se aplican a un gran número de muestras en una primera fase, y sólo las muestras no conformes se analizan de nuevo con los métodos de confirmación. En general, los métodos de cribado son menos costosos, menos específicos y más rápidos que los métodos de confirmación. Sin embargo, también se han desarrollado métodos de cribado basados en la espectrometría de masas con una preparación mínima de la muestra con el fin de proporcionar resultados fiables y rápidos.

Para los métodos de confirmación, la tasa de falso no conforme para las sustancias del grupo A al nivel de interés debe ser inferior al 1%, mientras que para las sustancias del grupo B, la tasa debe ser inferior al 5%. Las sustancias del grupo A deben analizarse mediante cromatografía de líquidos (LC) o cromatografía de gases (GC) con espectrometría de masas (MS) o espectroscopia de infrarrojos (IR) como sistema de detección, cumpliendo unos requisitos específicos. Las substancias del Grupo B se pueden analizar y confirmar utilizando LC con otros tipos de detectores, como por ejemplo UV/VIS de fila de diodos (DAD) o de fluorescencia.

Para la validación de un método de confirmación cuantitativo, deben determinarse o evaluarse parámetros tales como límite de decisión (CCα), capacidad de detección (CCβ), linealidad, veracidad/recuperación, precisión, selectividad/especificidad o la robustez.

La Decisión 2002/657/CE de la Comisión define el concepto de puntos de identificación (IP), que se utiliza se utiliza para establecer criterios de calidad para los métodos cualitativos y cuantitativos. La idea básica de los IP es que se permite que el laboratorio pueda aplicar cualquier técnica de espectrometría para confirmar la identidad de un

compuesto, mientras reúna un número mínimo de IP, necesarios para garantizar la correcta identificación del compuesto. Los métodos de confirmación basados en la detección MS tienen que sumar con 4 IP para substancias del grupo A (prohibidos) y 3 IP para las sustancias del grupo B (con LMR establecido). Además, el tiempo de retención debe coincidir con el del patrón del compuesto y la relación entre ion precursor e ion producto debe coincidir también con la del patrón, con unas tolerancias permitidas. Cuando el espectrómetro de masas es de alta resolución se utiliza cromatografía de líquidos acoplada un espectrómetro de masas de baja resolución, como un triple cuadrupolo (QqQ), con la monitorización de un ion precursor y dos iones producto de transición para cada analito es suficiente, pues obtienen suficientes IP (1 +1.5+ 1.5). En el caso de que el espectrómetro de masas sea de alta resolución es suficiente la medida de un ion precursor y de un ion producto (2 +2.5). Cabe mencionar que la Decisión 2002/657/CE no establece los criterios de exactitud de masa para la espectrometría de masas de alta resolución (HRMS), que tiene una gran repercusión en lo que se refiere a la confirmación de los compuestos. De hecho, para asegurar resultados fiables es necesario establecer un criterio de exactitud de masa con errores relativos en las medidas de masa inferiores a 5 ppm o en términos absolutos del orden de mDa es necesario establecer para proporcionar resultados de ensayos fiables.

En la UE, el Reglamento 882/2004 sobre los controles oficiales estableció una red de laboratorios que comprenden, los laboratorios comunitarios de referencia (CRL), los laboratorios nacionales de referencia (NRL) y los laboratorios oficiales de control (OCL). Los CRLs se encuentran en el nivel superior y entre sus principales responsabilidades están la organización de ensayos comparativos entre los laboratorios nacionales de referencia, el informar acerca de los avances en los métodos de análisis para laboratorios nacionales de referencia y el ofrecer asistencia técnica a la UE.

De acuerdo con el Reglamento CE 882/2004 todos los laboratorios de Europa que participan en los controles de seguridad de los alimentos deben estar acreditados por un organismo nacional de acreditación (por ejemplo ENAC en España) según la norma ISO/IEC 17025:2005. La norma es específica para la competencia de laboratorios de ensayo y calibración en todos los campos, incluyendo ensayos químicos, biológicos, ambientales, materiales y físicos. Consta de dos partes – los requisitos de gestión para garantizar el funcionamiento y la eficacia del sistema de gestión de la calidad, y los requisitos técnicos para garantizar la fiabilidad de las pruebas y calibraciones realizadas

en el laboratorio. La acreditación según la norma ISO / IEC 17025 se plantea según en el concepto de "analizado una vez, aceptado en cualquier parte".

Las metodologías analíticas consisten típicamente en el tratamiento de la muestra (extracción y/o *clean-up*), seguido de la separación cromatográfica y la detección de analitos. En el análisis de residuos de medicamentos veterinarios, la cromatografía líquida es la técnica de separación preferida y la espectrometría de masas la de detección, en especial con detectores QqQ .

La preparación de la muestra es una parte importante del método analítico, esencial para lograr resultados fiables y mantener el rendimiento del instrumento. El tratamiento de la muestra consiste en una etapa de extracción de los analitos, por lo general seguida de una etapa de *clean-up* para eliminar interferencias de la matriz. En algunas metodología destinadas al análisis de una serie de analitos prefijados, el tratamiento de muestra puede orientarse a la eliminación eficaz de la matriz y/o la preconcentración selectiva de analito.

Las técnicas de extracción más comúnmente utilizadas son la extracción líquido-sólido o la extracción líquido-líquido usando disolventes orgánicos y/o soluciones tampón acuosas dependiendo de la polaridad y pKa de los analitos. El acetonitrilo es uno de los disolventes de extracción preferidos ya que suele proporcionar una buena recuperación de analitos de variada polaridad y extraer bajos niveles de matriz. El metanol y el etanol son otros disolventes ampliamente utilizados. Las técnicas de extracción tales como la extracción asistida por microondas (MAE), extracción con fluidos supercríticos (SFE), o la extracción de líquido a presión (PLE) pueden resultar útiles cuando la extración con una simple agitación resulta insuficiente. Estas técnicas instrumentales también pueden ser ventajosas de cara a la automatización, o al aislamiento selectivo de residuos.

La modalidad de cromatografía de líquidos más empleada en este campo es la de fase inversa (RPLC). En RPLC la fase estacionaria es no polar (por ejemplo C18) y la fase móvil es polar, una mezcla agua/disolvente orgánico. La cromatografía de interacción hidrofílica (HILIC) es una modalidad que está ganando popularidad para el análisis de compuestos altamente polares. HILIC es una alternativa a la cromatografía en fase normal (NP). En HILIC la fase estacionaria es polar (por ejemplo, sílice) y la fase móvil es una mezcla de agua y disolvente orgánico, pero el agua es el eluyente más fuerte.

Por lo que se refiere a la espectrometría de masas, los analizadores de masas más ampliamente utilizados en el análisis de residuos son, tal como se ha mencionado anteriormente, los de baja resolución analizadores de triple cuadrupolo y se utilizan con frecuencia en el modo de monitorización de reacciones múltiples (MRM). El uso de analizadores de masas de alta resolución tales como el tiempo de vuelo (TOF) y orbitrap en el análisis de residuos está aumentando de forma importante en la actualidad, debido a la gran evolución experimentada en la instrumentación y al enrome potencial para la detección de numerosos analitos con altos niveles de confianza.

La tendencia actual en el ámbito de la metodología para el análisis de residuos, es el desarrollo de estrategias de análisis integrales basadas en la espectrometría de masas de alta resolución y que involucran el análisis *targeted* y *non targeted*. Esto se acelera debido principalmente a dos razones: i) el número de contaminantes a controlar en una muestra es cada vez mayor; ii) la instrumentación HRMS ha evolucionado y proporciona la sensibilidad y la solidez suficiente para el análisis a nivel de trazas. Las estrategias de análisis globales basadas en el cribado de amplio alcance de contaminantes o los estudios basados en -ómicas permiten proporcionar resultados de los análisis del tipo muestra segura/muestra no segura en contraposición a resultados del tipo si/no para unos residuos o contaminantes específicos.

En esta tesis, y en el marco del contexto anterior, se han planteado unos objetivos específicos relacionados con el ámbito del control de la seguridad alimentaria, que se presentan a continuación:

1. Desarrollo y validación de un método analítico basado en LC- UV para el análisis de ocho sulfonamidas en seis tipos de piensos.

2. Evaluación de diferentes fases estacionarias HILIC para el análisis de aminoglucósidos. Desarrollo y validación de métodos analíticos para la confirmación y cuantificación de diez aminoglucósidos en muestras de riñón y en miel.

3. Exploración de los diferentes modos de adquisición de un espectrómetro de masas híbrido cuadrupolo Orbitrap para análisis específicos y desarrollo de un método para analizar nueve hormonas en muestras de orina de animales.

4. Estudio de resultados falso no conforme de ronidazol en carne de un método LC-QqQ-MS/MS y aplicación de LC-MS/HRMS.

5. Desarrollo de un *workflow* para una estrategia de análisis *non targeted* usando datos de LC-MS/HRMS y herramientas de análisis de datos.

Aparte del método de análisis de sulfonamidas y de los estudios sobre análisis *non targeted*, el resto de métodos han sido desarrollados en colaboración con el Laboratori de l'Agència de Salut Pública de Barcelona, en el marco de un convenio entre esta entidad y la Universitat de Barcelona. Los métodos han sido validados de acuerdo con la Decisión 2002/657/CE y se aplican en el análisis rutinario de conformidad con la norma ISO 17025:2005 .

En el Capítulo 4 se presenta un método analítico basado en LC-UV para analizar las sulfonamidas en piensos. Las sulfonamidas son un tipo de antibióticos sintéticos que tienen un grupo funcional $-SO_2NH_2$. Las sulfonamidas son compuestos anfóteros, con dos equilibrios ácido base; la forma neutra, que es la más soluble en disolventes orgánicos, predomina entre pH 3 y 6 aproximadamente. Con un control adecuado del pH se pueden extraer selectivamente las sulfonamidas, o diseñar procesos de clean-up, utilizando sorbentes de extracción en fase sólida adecuados.

Las sulfonamidas son bacteriostáticos que inhiben el crecimiento y la multiplicación de las bacterias y son el grupo de antimicrobiano más antiguo. La primera sulfonamida (sulfanilamida) fue sintetizado en 1936. Hoy en día, las sulfonamidas se utilizan en la agricultura, la acuicultura y la cría de animales y raramente en la medicina humana. Desde 2006 en la UE está prohibido el uso de sulfonamidas, y antibióticos en general, como promotores del crecimiento en animales productores de alimentos. Sin embargo, las sulfonamidas están autorizadas para su uso en piensos medicados con fines terapéuticos.

La presencia de antimicrobianos en los piensos puede ser debida a su uso autorizado con propósito terapéutico o profiláctico, a su uso no autorizado como promotores del crecimiento para aumentar el rendimiento, o a la contaminación cruzada no intencionada que se produce en el lugar de producción, donde piensos medicados y no medicados se preparan en la misma línea de producción. La contaminación cruzada es inevitable, incluso después de la aplicación de buenas prácticas de fabricación, y por tanto una razón importante para la presencia de sulfonamidas en los piensos. El control de la presencia de sulfonamidas es relevante en este tipo de muestras, ya que plantean

riesgos para la seguridad alimentaria, en especial el relacionado con la resistencia a los antibióticos cuando se introducen en la cadena alimentaria.

Los piensos son una matriz compleja y su composición puede variar enormemente entre diferentes tipos de piensos. La composición varía dependiendo del tipo de animales a los que van destinados y también dependiendo de la fase de crecimiento del animal.

En este trabajo, se desarrolló un método basado en LC-UV sencillo para la determinación de residuos de ocho sulfonamidas (sulfacloropiridazina, sulfadiazina, sulfadoxina, sulfametoxipiridazina, sulfaquinoxalina, sulfametoxazol y sulfadimetoxina). Se evaluaron cartuchos de SPE de las fases C18, Oasis HLB, plexos y Plexa PCX para la etapa de limpieza. Se seleccionó la fase Plexa PCX ya que mostró una mayor eficacia en la limpieza de las interferencias. Se ensayaron seis tipos de pienso (cerdo, cochinillo, pollo, gallina, ternera y conejo). Los estudios sobre los efectos de matriz, comparando las pendientes de las funciones de calibración, mostraron que el pienso de ternera y el de pollo son los que muestran más influencia. El método fue validado y en la tabla 2 se resumen los parámetros de calidad del método.

En el capítulo 5 de presenta un estudio en el que se evalúan fases estacionarias y fases móviles para la separación de aminoglucósidos mediante cromatografía de líquidos de interacción hidrofílica (HILIC), así como la optimización y validación de métodos basados en LC-MS para el análisis de diez aminoglucósidos en muestras de riñón y de miel.

Tabla 2. Resumen de parámetros de calidad del método LC-UV para él análisis de sulfonamidas en piensos.

LOD (en la muestra)	74-265 µg/kg
LOQ (en la muestra)	265-868 µg/kg
Recuperación de la extracción	47-66%
Precisión intradía	4 – 15%
Precisión interdía	7-18%

Los aminoglucósidos son una clase de agentes antimicrobianos que se administran a los animales productores de alimentos para el tratamiento de diversas enfermedades. Su

presencia en los animales debe ser controlada debido a riesgos relacionados principalmente con su ototoxicidad y nefrotoxicidad. Además, como todos los antibióticos, su uso excesivo o inadecuado puede causar resistencia a los fármacos antimicrobianos.

Los aminoglucósidos se componen de glucósidos unidos con grupos amino ($-NH_2$). Tienen varios grupos ionizables y son altamente polares. Por ello su separación mediante cromatografía de líquidos de fase inversa requiere el uso de agentes formadores de pares iónicos, como por ejemplo el ácido heptaflourobutírico (HFBA), para lograr la retención. En presencia del reactivo y con las condiciones adecuadas, los aminoglucósidos forman los pares iónicos que interaccionan con la fase estacionaria de fase inversa, y de este modo es posible lograr la separación cromatográfica. Pero esta estrategia presenta limitaciones importantes, debido a la fuerte supresión iónica en la detección MS causada por el reactivo utilizado para la formación de los iónicos y también se requiere la limpieza intensiva y frecuente del espectrómetro, para eliminar depósitos y residuos.

Una alternativa a la cromatografía de líquidos en fase inversa de pares iónicos de los aminoglucósidos es la modalidad HILIC. HILIC es una variante de la cromatografía de fase normal, en la se produce retención de compuestos altamente polares. Las fases estacionarias pueden agruparse en neutras, polares o iónicas, en función de las variaciones estructurales de los grupos funcionales. En la última década, la modalidad HILIC ha experimentado un gran progreso y se ha utilizado en muchas aplicaciones. Los tipos de fases estacionarias HILIC disponibles comercialmente van aumentando.

Los mecanismos de retención y de separación en HILIC dependen del grupo funcional de la fase estacionaria, de las características de fase móvil (contenido de agua, el pH, fuerza iónica) y las propiedades de los analitos. El mecanismo de retención del analito es multimodal (figura 2) con la contribución de:

i) partición entre la capa de agua formada en la fase estacionaria y la fracción orgánica de la fase móvil

ii) intercambio iónico/interacciones electrostáticas entre el analito ionizado y la fase estacionaria

iii) la adsorción.

A menudo es difícil predecir el mecanismo de retención predominante en HILIC. Con el fin de conocer cuál es el predominante para los analitos estudiados y poder predecir la retención y la separación en diversas fases estacionarias, es habitual utilizar modelos teóricos o empíricos durante el desarrollo del método. Por ejemplo, en el caso de los aminoglucósidos, cuando la fuerza iónica de la fase móvil aumenta, el intercambio de iones disminuye, y cuando el pH de la fase móvil disminuye, las interacciones con los grupos silanol disminuyen, de modo que la partición pasa a ser el mecanismo más dominante.

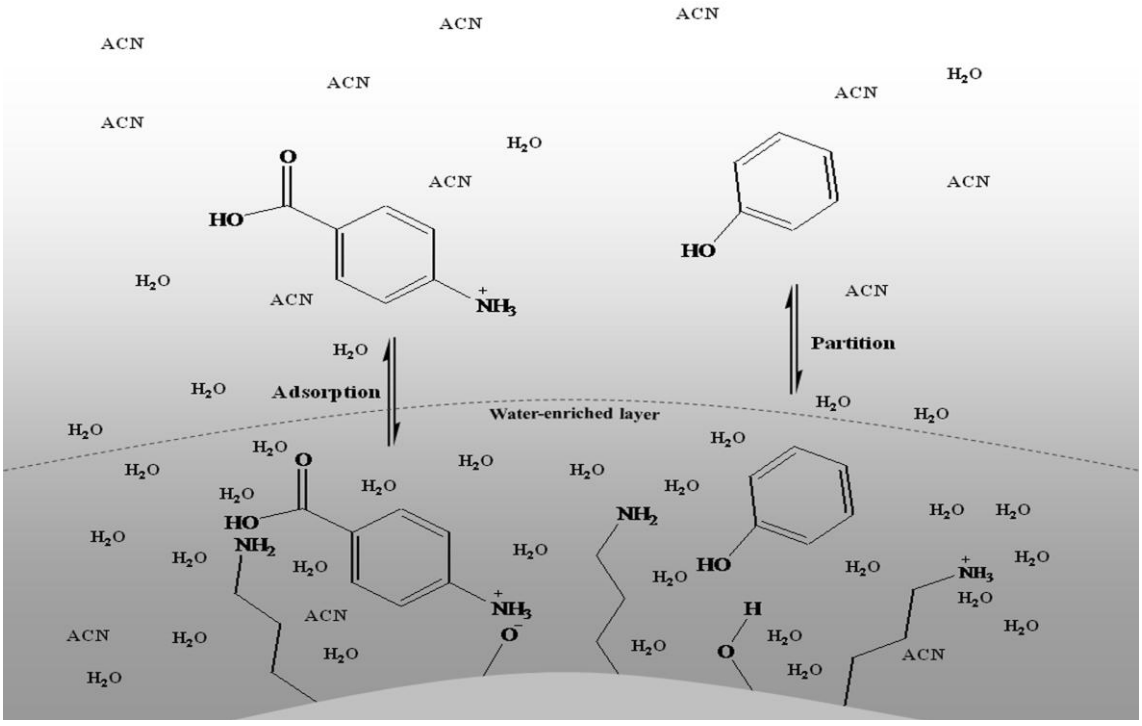

Figura 2. Mecanismos de retención multimodales entre una fase estacionaria HILIC y la fase móvil. Reproducido con permiso de Nguyen y Schug

Es muy difícil de incluir aminoglucósidos en métodos analíticos de residuos de fármacos multifamilia ya que, al ser compuestos muy polares, sus propiedades químicas son considerablemente diferentes a las de las otras familias. En ese sentido, tiene interés el desarrollo de un método dirigido exclusivamente al análisis de aminoglucósidos. En concreto los objetivos de este trabajo fueron:

- Estudio de diversas fases estacionarias HILIC para la separación cromatográfica de los aminoglucósidos.

- Optimización de las condiciones de la fase móvil para retener y separar aminoglucósidos.
- Desarrollo y validación de metodologías analíticas para el análisis de diez aminoglucósidos (estreptomicina, dihidroestreptomicina, espectinomicina, apramicina, paromomicina, kanamicina A, gentamicina C1, gentamicina C2/C2a, gentamicina C1a y neomicina) en muestras de riñón y de miel.

En este trabajo, se estudió de manera sistemática, mediante un diseño central compuesto central, el efecto de las condiciones de fase móvil (pH y la fuerza iónica) sobre el comportamiento de los diez aminoglucósidos en columnas de HILIC de sílice, amino, amida y zwitteriónica. Esta última fase fue la que proporcionó una mejor separación de los aminoglucósidos. Se desarrollaron dos métodos basados en HILIC y con detección MS/MS con un equipo de triple cuadrupolo para la determinación de aminoglucósidos en muestras de riñón y de miel. Los métodos constan de la extracción de los analitos con una solución acuosa (para muestras de riñón) o la disolución de la muestra en agua (para muestras de miel), la purificación de las disoluciones obtenidas, mediante extracción en fase sólida con un cartucho de intercambio catiónico débil y la inyección del eluato en el sistema cromatográfico. Las paramétros de calidad del método se resumen en las tablas 3 y 4. Los valores CCα y CCβ de aminoglucósidos en el método para el análisis de miel son más bajos que los valores en el método para el análisis de riñón. Esto se debe a que los valores de LMR para los aminoglucósidos en el riñón son relativamente altos, mientras para miel que no hay LMR establecidos y se aplica el principio tolerancia cero.

Tabla. 3. Resumen de las características de funcionamiento del método de análisis de aminoglucósidos en miel.

CCα	13-64 µg/kg
CCβ	17- 99 µg/kg
LOQ (en la muestra)	2 – 125 µg/kg
Veracidad (expresada como sesgo)	7-20%

Precisión interdía a concentraciones bajas (20-70 µg/kg)	12-26%

Tabla 4. Resumen de las características de funcionamiento del método para el análisis de aminoglucósidos en riñón

CC α	798-22864 µg/kg
CC β	846-25728 µg/kg
LOQ (en la muestra)	25-264 µg/kg
Veracidad (expresada como sesgo)	1-11 %
Precisión interdía a concentraciones bajas (200-300 µg/kg)	9-21 %

Aunque estos métodos desarrollados con HILIC evitan el uso de agentes de formadores de pares iónicos, y proporcionan buenos resultados en aplicación al análisis de rutina y en pruebas de competencia, se utiliza una fase móvil acuosa de elevada fuerza iónica (175 mM), que puede ocasionar efectos importantes de supresión iónica en la detección MS. Debido a esto algunos autores argumentan a favor del uso de la cromatografía de pares iónicos en lugar de HILIC.

Por otra parte, alguno de los picos que eluyen al final del cromatograma, como la neomicina, presentra cola, que se intensifica tras múltiples inyecciones de muestra en la columna. A partir de nuestra experiencia al presentar el trabajo anterior en congresos y tras su publicación, quedó claro que muchos laboratorios estaban interesados en disponer de un método robusto para el análisis de los aminoglucósidos, que utilice una fase móvil simple y que evite problemas de *crosstalk* que pueden producirse cuando se utiliza detección MS/MS de baja resolución.

De la experiencia previa adsquirida durante la evaluación de distintas fases estacionarias para HILIC, la fase tipo amida es una buena candidata para el análisis de aminoglucósidos, pues ofrece la posibilidad de utilizar una fase móvil simple que

proporciona picos de simetría adecuada, aunque no se separan todos los analitos. De todos modos esta última no una limitación importante cuando se utiliza una detección MS/MS. Por otro lado, la detección con un equipo Q-Orbitrap HRMS ofrece la posibilidad de utilizar iones precursores para la cuantificación, evitando así el *crosstalk* entre iones producto idénticos. En esa dirección, se desarrolló un método HILIC-MS/HRMS, basado en una separación con una fase estacionaria con el grupo funcional amida y que utiliza como detector un espectrómetro de masas híbrido Q – Orbitrap; ello conduce a la obtención de picos cromatográficos de simetría adecuada y evita problemas de *crosstalk* en la detección mediante espectrometría de masas.

En el Capítulo 6 se presenta un estudio que explora los diversos modos de adquisición y los parámetros de un espectrómetro de masas de cuadrupolo híbrido-orbitrap y un método basado en cromatografía de líquidos - espectrometría de masas (Q-Orbitrap) para la determinación de ocho hormonas sintéticas (trembolona, 17α etinilestradiol, zeranol, estanozolol, dienestrol, dietilestilbestrol, hexestrol, taleranol) y una hormona natural (zearalenona) en muestras de orina de animales.

El Q-Orbitrap (Q-Exactive) es un instrumento híbrido de alta resolución. Fue introducido por primera vez en 2011 y se aplica generalmente con fines de investigación en los campos de la metabolómica, proteómica, seguridad alimentaria y análisis ambiental. Sin embargo, su implantación en los laboratorios oficiales de control es por el momento escasa. De hecho, los espectrómetros de masas de triple cuadrupolo siguen siendo los caballos de batalla en el campo de los laboratorios de análisis en este ámbito debido a su coste, facilidad de uso y robustez. Hasta no hace muchos años los instrumentos de HRMS eran menos robustos (requerían calibraciones frecuentes y un elevado nivel de mantenimiento) y, a menudo, no proporcionaban la sensibilidad suficiente como para satisfacer las necesidades de los laboratorios de análisis de alimentos. La nueva generación de instrumentos Orbitrap ha aumentado la eficiencia de transmisión de la corriente de iones, lo que lleva a una alta sensibilidad. Por otra parte, la presencia de un cuadrupolo en el instrumento híbrido Q-Orbitrap, para filtrar interferencias de la matriz no deseadas y aislar iones precursores, junto con la opción de multiplexación, para aumentar la velocidad de exploración, proporcionan unas características de funcionamiento muy interesantes para el análisis de alimentos. Así, la combinación de los elevados poder de resolución, exactitud en la medida de la masa y

sensibilidad, junto con la adquisición de espectros, abre nuevas posibilidades en los análisis relacionados con la seguridad de los alimentos.

La presencia del cuadrupolo ofrece diferentes modos de adquisición de datos, destacando especialmente las adquisiciones dependientes (DDA). Para lograr unos resultados óptimos, tienen que ser optimizados varios parámetros del espectrómetro de masas. En ese sentido, se consideró necesario realizar un estudio sistemático con algunos analitos modelo en una matriz compleja para identificar los parámetros clave y averiguar cómo optimizarlos para llevar a cabo análisis de rutina. Así, para llevar a cabo estos estudios se eligió la determinación de hormonas en orina, que se trata de un problema analítico de elevada complejidad, a causa especialmente de la matriz. El laboratorio de la ASPB había desarrollado anteriormente un método basado en LC con detección MS/MS mediante un instrumento de triple cuadrupolo, y se trataba de poner a punto el método LC-Q-Orbitrap.

Los agentes anabolizantes, tales como hormonas y otras sustancias hormonalmente activas, se han utilizado en las prácticas de la ganadería intensiva desde la década de 1950 para aumentar la tasa de crecimiento de los animales. Actualmente su uso está regulado o prohibido en muchos países, debido al posible carácter carcinogénico de los residuos de estas sustancias en los alimentos de origen animal.

Se pueden administrar como aditivos para piensos, mediante inyección o con implantes que liberan lentamente el producto, aunque la primera opción es la más común. Los agentes anabolizantes se metabolizan y excretan rápidamente y por ello se pueden encontrar concentraciones elevadas en la bilis, orina y heces. Así, la orina es una muestra ideal para el control de estos compuestos.

Desde una perspectiva analítica, la orina es una matriz difícil, con una gran cantidad de posibles interferencias, y por ello permite explorar a fondo las capacidades de los equipos de HRMS. El objetivo principal de este trabajo ha sido evaluar el rendimiento de la instrumentación Q-Orbitrap para análisis *targeted* y por otro lado la transferencia a la modalidad LC-Q-Orbitrap-MS/MS de un método basado en LC-QqQ-MS/MS para el análisis de hormonas sintéticas en orina, previamente desarrollado en el Laboratorio de la ASPB. En la Tabla 5 se resumen las características de funcionamiento del método.

Usando los modos de adquisición con cuadrupolo, el sistema de MS Orbitrap proporciona la sensibilidad requerida para el análisis de residuos. Además, la capacidad de este tipo de instrumentación para adquirir espectros completos proporciona la posibilidad de analizar de forma retrospectiva los datos para el análisis *non targeted*, que es una estrategia muy prometedora en el campo de la seguridad alimentaria.

Tabla 5. Características de funcionamiento del método LC-Q-Orbitrap-MS/MS para el análisis de hormonas sintéticas en la orina.

CCα	0.11 – 0.69 μg/l
CCβ	0.33 – 0.90 μg/l
Veracidad (expresada como sesgo)	1.4 – 3 %
Precisión interdía a concentraciones bajas (1 μg/l)	4.9 – 18.2 %
Recuperación de la extracción	65 – 83 %

En el Capítulo 7 se presenta un caso de falsos positivos debido a la interferencia de la matriz en el análisis de ronidazol en tejido muscular. En la mayoría de los laboratorios de análisis de alimentos, los espectrómetros de masas QqQ son los instrumentos más utilizados para confirmar la presencia de contaminantes en las muestras. Sin embargo, la resolución que proporciona esta instrumentación no siempre es suficiente para resolver interferencias isobáricas que coeluyen en el análisis de alimentos con matrices complejas, lo que afecta a la fiabilidad de los resultados. En esta línea hay evidencias previas de falsos no conformes de residuos sebutilazina o de nitroimidazol, o falsos conformes de benzofenona, lo que pone de manifiesto la potencial utilidad de la espectrometría de masas de alta resolución (HRMS) en este ámbito. El falso no conforme de sebutilazine se debía a un compuesto isobárico y endógeno (nepllitorine) en la matriz de estragón, lo que provocó una variación significativa en la ratio de iones, y finalmente se resolvió mediante LC -TOF. Los falsos conformes de benzofenona se debían a una interferencia isobárica, que provocaba una desviación en el criterio del ratio de iones más allá del rango de tolerancia, lo que lleva a la interpretación como un resultado negativo. El problema se resolvió mediante un espectrómetro de masas Orbitrap (Exactive) y se pudo comprobar que se trataba de un falso conforme.

La Decisión 2002/657/CE de la UE establece los criterios de confirmación basados en el concepto de puntos de identificación (IP). De acuerdo con la presente Decisión, se

requieren cuatro puntos de identificación para confirmar la presencia de sustancias prohibidas: un punto por un ión precursor y 1.5 puntos por cada producto de transición en el caso de un espectrómetro de masas de baja resolución (como por ejemplo el analizador de triple cuadrupolo). Además, se debe controlar al menos un ratio de iones y se deben cumplir los criterios de tolerancia para este parámetro, en función de las intensidades relativas de los iones.

La Decisión 2002/657/CE establece criterios específicos para el uso de la HRMS, con respecto a puntos de identificación al monitorizar iones específicos, pero no hace ninguna referencia sobre los criterios de precisión en la medida de masas, lo que es una limitación importante de este documento. Algunos autores han puesto sobre la mesa la necesidad de actualizar los criterios para el uso de instrumentación HRMS y han propuesto un conjunto de directrices. La Comisión del Codex Alimentarius está elaborando un documento de directrices actualizadas sobre las características de funcionamiento de los métodos analíticos multirresiduo, y propone que el exactitud en la medida de masa cuando se utiliza HRMS instrumentación debe ser inferior a 5 ppm.

El ronidazol pertenece a la familia de antibióticos de los nitroimidazoles. Su uso en animales productores de alimentos está prohibido en la UE por tres Reglamentos, 3426/93/EC, 1798/95/EC y 613/98/EC . Se han publicado varios métodos basados en LC-MS para analizar residuos de ronidazol en muestras de músculo.

En estudios llevados a cabo en esta tesis, se comprobó que el resultado no conforme del análisis de rodinazol mediante LC-QqQ-MS/MS en una muestra de músculo y que cumplía todos los criterios de confirmación de la Decisión 2002/657/CE, era en realidad un falso no conforme, tras su análisis mediante LC–HRMS.

Cuando la muestra se analizó usando como detector un instrumento HRMS (Q-Orbitrap) trabajando a una resolución elevada (70,000 FWHM), este resultado falso no conforme se comprobó que era debido a interferencias de la matriz isobáricas . Este caso demuestra claramente la utilidad de la instrumentación HRMS en laboratorios de seguridad alimentaria, con el fin de evitar resultados falsos positivos derivados de interferencias de la matriz . En la bibliografía cada vez se describen más casos de falsos no conformes cuando se utilizan instrumentos de baja resolución, como el QqQ. Para evitar estos problemas y aumentar la selectividad, podría ser conveniente controlar un tercer ion producto y una segunda ratio de iones.

Como se resultado de los estudios llevados a cabo, en el laboratorio se establecieron criterios para el análisis de confirmación con HRMS, que durante los últimos seis meses el laboratorio de la ASPB ha estado aplicando en las muestras analizadas mediante LC-HRMS. Los criterios establecidos son, tolerancia en el tiempo de retención de ± 1 %, al menos un ion producto a elevada resolución (> 20,000 FWHM), una resolución mínima de 70,000 FWHM para los iones precursores, exactitud en la medida de masa inferior o igual a 5 ppm, y el seguimiento de al menos una ratio de iones. La calibración del Orbitrap cada 3 días es suficiente para asegurar la exactitud de masa inferior a 5 ppm.

En el capítulo 8 se presenta un *work flow* para análisis *non targeted* basado en una estrategia de consulta de bases de datos de espectrometría de masas de cuadrupolo-Orbitrap.

En los campos del análisis ambiental y del de residuos y contaminantes en alimentos, los métodos de análisis se desarrollan para determinar o detectar contaminantes pertenecientes a una o pocas familias (análisis *target*), que son las que solicitan o indican los organismos encargados del control. Sin embargo, existe una alta probabilidad de que estén presentes otros contaminantes pertenecientes a diferentes familias químicas, como los pesticidas, toxinas naturales, contaminantes industriales, contaminantes procedentes de los materiales de empaquetamiento, etc. que se pueden introducir en cualquier punto de la cadena alimentaria, desde la granja hasta la mesa. Esto exige un cambio en los métodos de análisis, para pasar de proporcionar una respuesta de tipo binario “residuos no presentes (conforme) / residuos presentes (no conforme)”, a análisis integrales con respuesta del tipo “muestra segura/ muestra no segura”. Desde una perspectiva analítica esto implica el desarrollo de estrategias de análisis *non targeted.*

Los instrumentos basados en analizadores Orbitrap han evolucionado rápidamente en los últimos años y el instrumento híbrido cuadrupolo-Orbitrap (Q Exactive) proporciona unas excelentes características de funcionamiento, tales como el gran poder de resolución, medidas de masa exactas, adquisición de espectros y la presencia del cuadrupolo para aislar precursores en el análisis de muestras con matrices complejas, tales como las que podemos encontrar en el análisis de residuos y contaminantes en alimentos o en el análisis de contaminantes en muestras ambientales.

El análisis integral de una muestra implica un paso genérico de extracción, la separación cromatográfica y la detección por espectrometría de masas de alta resolución seguido por análisis *targeted* y *non targeted*. El análisis *targeted* es para sustancias para las que el método ha sido validado con patrones. El enfoque de detección de *suspects* está dirigido a los compuestos para los que se dispone de algo de información previa. Por último el análisis *non targeted* se centra en las sustancias para los que no se tiene ninguna información previa. Hay que mencionar que las estrategias de análisis *non targeted* basadas en la consulta de bases de datos dependen en gran medida de las bibliotecas espectrales y en disponer de las herramientas de análisis de datos adecuadas.

Aunque diferentes autores utilizan diferentes términos para el análisis *non targeted*, la clasificación basada en el *workflow* conduce a dos categorías - *targeted* y *non targeted*. Tras identificar un compuesto este se puede en tres categorías diferentes- *known knowns*, *known unknown* y *unknown unknowns* . Los compuestos *known knowns* son los analitos *target*. Los *known unknowns* son los compuestos inesperados por el analista, pero identificados en las bibliotecas integrales de datos. Los *unknown unknowns* son los compuestos inesperados por el analista y no identificados en las bibliotecas de datos. Desde la perspectiva de un laboratorio de control, puede ser de gran utilidad la creación de una base de datos recurrente para *unknown unknowns* detectados, y tratar de elucidar la estructura de los que estén presentes con frecuencia en las muestras analizadas.

Tal como se describe en el capítulo 4, los datos obtenidos con instrumento Q-Orbitrap pueden proporcionar la masa exacta medida, el aducto, el patrón isotópico y el MS/MS del ión precursor aislado fragmentado a diferentes energías de colisión para cada sustancia química ionizada. En el análisis *non targeted* el puzzle es identificar el compuesto a partir de estas características.

Esto implica el uso de diversas herramientas de análisis de datos. En este escenario, el objetivo de este trabajo ha sido el desarrollo de un *workflow* de análisis de datos para el análisis *non targeted* para los datos obtenidos con ionización negativa, y aplicarlo a los datos obtenidos en una muestra de sedimento. Este trabajo se ha llevado a cabo durante una estancia de investigación en el grupo de Química Ambiental del EAWAG (Instituto de Investigación Federal Suizo). Se ha desarrollado un *workflow* sistemático para el análisis *non targeted* con LC-MS/HRMS (Q Exactive) para picos *non targeted* que

continen Cl, Br. Los compuestos que contienen Cl y Br representan el 19.2 % de los compuestos registrados en la base de datos Chemspider. El *workflow* se puede aplicar además a otros compuestos.

En la muestra de sedimento estudiada dos picos *known unknows* se han identificado y confirmado con patrones como hexaclorofeno y flucofuron. Este *workflow non targeted* se puede aplicar a cualquier campo de análisis, incluidos los métodos de análisis relacionados con la seguridad alimentaria que utilizan un analizador Orbitrap.

Conclusiones de la tesis:

Se ha desarrollado un método sencillo, basado en LC – UV, para el análisis de sulfonamidas en piensos.

Se han explorado de manera sistemática diversas fases HILIC para la retención cromatográfica de aminoglucósidos y se han desarrollado métodos HILIC -MS/MS para el análisis de aminoglucósidos en muestras de miel y de riñón. Por otra parte, se ha desarrollado un método cromatográfico robusto y simple basado en HILIC-MS/HRMS.

Se ha explorado el potencial de un instrumento híbrido cuadrupolo-Orbitrap en el ámbito del control de la seguridad alimentaria. Se han investigado a fondo diferentes modos de adquisición de espectros y se ha puesto a punto un método para el análisis de hormonas en orina.

Se ha resuelto con LC-MS/HRMS un resultado falso no conforme proporcionado por un método LC-MS/MS de baja resolución. Se identificó que la asignación errónea de un pico cromatográfico se debía a interferencias isobáricas de la matriz, que podían ser claramente resueltas con medidas de masa mediante HRMS. Esto también pone de relieve la necesidad de establecer criterios de confirmación adecuados para los resultados de los análisis.

Se ha desarrollado un *workflow* de análisis *non targeted* utilizando diversas herramientas de minería de datos y de análisis, que se ha aplicado a una muestra de sedimento. En el sedimento se han identificado algunos compuestos *suspect* y dos compuestos *unknown*.

Cover image

An integrated agroforestry and animal farming system based on natural farming philosophy. This system intends to avoid the use of agrochemicals and ensure safe and sustainable food supply.

vs

A conventional modern food chain comprising monoculture of plants and industrial animal farming, with the aid of agrochemicals such as chemical fertilizers, pesticides, veterinary drugs and growth promoters. Safe food supply is ensured by a centralized detective control system.